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"ALLITHIAMINE", A NEWLY FOUND DERIVATIVE OF VITAMIN B₁

IV. ON THE ALLIIN HOMOLOGUES IN THE VEGETABLES

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(Received for publication, May 16, 1957)

In the first report (1) Fujiwara, one of the authors, stated that thiamine was changed into allithiamine by the chemical reaction with allicin contained in the garlic extract and that this phenomenon was observed in the other plants of allium species than garlic, such as *Allium odorum*, *Allium Bakeri* Regel etc. In the previous report (2) the authors reported the results of the researches into allicin homologues in the plants of allium species by the paper chromatographic method.

In the course of studying alliin homologues in the plant of allium species, connected with the investigations of allicin, the authors have found the interesting phenomena in some other plants than allium species, as itemized below (3).

1. Pyruvic acid and ammonia are produced in the extract of the root of *Ipheion uniflorum* Rafinesque (called *Hananira* in Japanese), when this extract is mixed with protein of garlic.

2. Allithiamine homologues are produced when thiamine is mixed in the resultant mixture.

3. These similar phenomena shown in *Ipheion uniflorum* above mentioned are detected in the cases of radish, cabbage etc.

In order to throw light on the mechanisms of these phenomena, a study has been made on the ingredients of these plants. It has reached the conclusion that the other plants than allium species contain the same intrinsic alliin homologues as allium species do, but they do not contain allinase.

The present papers report the results of the studies drawing the above conclusion and the distribution of alliin homologues in the vegetable kingdom which hitherto we have investigated.

Now we will discuss allicin homologues with the general formula of $R-S-S-R$ and alliin homologues with $R-S-\overset{\overset{O}{\parallel}}{C}-CH(NH_2)-COOH$, in which

R represents an alkyl radical. When R is CH_3 it is commonly called methylalliin, for example.

METHODS

Paper Chromatography of Alliin Homologues ($\text{R}-\text{S}-\overset{\text{O}}{\underset{\text{O}}{\text{C}}}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$)—Two-dimensional

chromatography was run on a sheet of 40×40 cm filter paper (solvents, phenol ammonia (8:2) and *n*-butanol-acetic acid-water (4:1:5)) in the ordinary manner. The chromatograms were dried with a stream of warm air (80–90°) after a ninhydrin solution had been sprayed. The R_f values of the synthesized alliin homologues are listed in Table I.

TABLE I
 R_f Values of Alliin Homologues

Material	R_f values
Methyl-alliin	0.55, 0.15
Ethyl-alliin	0.60, 0.15
Alliin	0.67, 0.25
Propyl-alliin	0.67, 0.25

The spot of methyl-alliin is quite near that of arginine, so it seems difficult to distinguish the difference between these two materials only from paper chromatography. The spots of alliin and propyl-alliin are located in the same place.

Paper Chromatography of Allicin Homologues ($\text{R}-\text{S}-\overset{\text{O}}{\underset{\text{O}}{\text{C}}}-\text{S}-\text{R}$)—Paper chromatography of allicin

homologues was run in the same method as described in the previous report (2).

Differentiation of Alkyl Radicals in Allicin Homologues ($\text{R}-\text{S}-\overset{\text{O}}{\underset{\text{O}}{\text{C}}}-\text{S}-\text{R}$)—To differentiate the

alkyl radicals of allicin homologues the same method as described in the previous report (2) was used.

Paper Chromatography of Allithiamine—A paper (40 cm. in length, 1.5 cm in width) was used and developed with a *n*-butanol-acetic acid-water solution (4:1:5) by the descending method. After drying, a solution of 200 mg. per cent cysteine-HCl was sprayed and heated at 80° for 15 minutes, prior to the spraying of potassium ferricyanide solution (1 per cent potassium ferricyanide-30 per cent NaOH- water (3:2:7)). The fluorescences were examined with an ultraviolet lamp in the dark room. When the spraying of a cysteine- HCl solution is omitted, no fluorescence of thiochrome converted from al-

TABLE II
 R_f Values of Derivatives of Thiamine etc.

Material	R_f values
Allithiamine	0.85
Propyl-allithiamine	0.85
Methyl-allithiamine	0.75
Thiamine	0.30
Thiochrome	0.50
Thiamine disulfide	0.35

lithiamine appears. The R_f values of thiamine, allithiamine, thiochrome, etc. are listed in Table II.

Determination of Pyruvic Acid—For the quantitative determination of pyruvic acid the method of Friedmann and Haugen (4) was applied.

Determination of Ammonia—For the quantitative determination the azotometry which was devised by Iwasaki (5) was applied.

RESULTS

I. *Ipheion uniforum* Rafinesque

Pyruvic Acid and Ammonia in the Extract of Ipheion uniforum—The water extract of ground fresh *Ipheion uniforum*, centrifuged at 3000 r.p.m. for 20 minutes, was a turbid fluid. A portion of its supernatant part, 2 ml., was mixed with 2 ml. of water suspension of protein obtained from garlic. (The specimen of protein is obtained from the precipitate formed in the extract of fresh garlic, when it is acidified to pH 4. The precipitate is washed three times with distilled water.) Pyruvic acid and ammonia in the resultant mixture were determined. As a control the same examination was performed with the extract of *Ipheion uniforum* which was mixed with the boiled extract of the protein of garlic or to which the protein of garlic was not added. The results are shown in Table III.

TABLE III

Pyruvic Acid and Ammonia in the Extract of Ipheion uniforum

Material	Pyruvic acid (mg %)	Ammonia (mg %)
Extract of <i>Ipheion uniforum</i> +protein of garlic	426	84
Extract of <i>Ipheion uniforum</i> +boiled protein of garlic	0	0
Extract of <i>Ipheion uniforum</i>	0	0

As shown in the Table III the marked appearances of pyruvic acid and ammonia are observed in the mixture of the extract of *Ipheion uniforum* with the protein of garlic. On the contrary there is no appearance of pyruvic acid and ammonia in the mixture of the extract of *Ipheion uniforum* with the boiled protein of garlic, or in the extract of *Ipheion uniforum* only. These facts suggest that pyruvic acid and ammonia are produced from the constituent of *Ipheion uniforum* by the action of enzyme of garlic.

Production of Allithiamine Homologues by the Extract of Ipheion uniforum—As it was expected that in the resultant mixture above mentioned allicin or its homologues might be produced, thiamine was added to the mixture. Then the mixture was allowed to stand for 30 minutes in the water bath at 60° after the pH had been adjusted to 8. Thereupon, it was detected that thiamine added to this mixture lost its thiochrome reaction. So an experiment was carried out for the explanation of the above thiamine which lost

TABLE IV

R_f Values of Allithiamine Homologues in the Extract of Ipheion uniforum

Material	<i>R_f</i> values			
Mixture of the extract of <i>Ipheion uniforum</i> , protein of garlic and thiamine (pH 8) heated at 60° for 30 minutes.	0.30*,	0.50*,	0.75,	0.85
Extract of <i>Ipheion uniforum</i> mixed with thiamine (pH 8) heated at 60° for 30 minutes.	0.30,	0.50		
Thiamine	0.30			
Allithiamine				0.85
Methyl-allithiamine			0.75	
Thiochrome		0.50		
Thiamine-disulfide	0.35			

* very faint spot.

its thiochrome reaction with paper chromatograph. The results of this experiment show that this thiamine is converted to one of allithiamine homologues. The results of the paper chromatograph are shown in Table IV.

Paper Chromatograph of Allicin Homologues in the Extract of Ipheion uniforum mixed with Protein of Garlic—From the fact that the allithiamine homologues are produced in the mixture above mentioned, it may be inferred that allicin or its homologues is produced in the mixture of the extract of *Ipheion uniforum* with the protein of garlic. So, further investigation was made by the paper chromatographic method (see Method) in order to ascertain these reaction products. The following are the results.

Three spots of allicin homologues at *R_f* 0.12, 0.65–0.75 and 0.9, were found from the mixture of the extract of *Ipheion uniforum* with the protein of garlic, but such a spot was not found from the extract of *Ipheion uniforum* without any addition of the protein of garlic. The second spot at *R_f* 0.65 extended vaguely to 0.75. Then their alkyl radicals were examined. Only one spot at *R_f* 0.5, corresponding to $\text{CH}_3\text{-S-S-CH}_2\text{-CH(NH}_2\text{)-COOH}$, was found from the extract of a strip of the paper with *R_f* 0.12, three spots at *R_f* 0.5, 0.63 and 0.76, respectively $\text{CH}_3\text{-S-S-CH}_2\text{-CH(NH}_2\text{)-COOH}$, $\text{C}_2\text{H}_5\text{-S-S-CH}_2\text{-CH(NH}_2\text{)-COOH}$ and $\text{C}_3\text{H}_7\text{-S-S-CH}_2\text{-CH(NH}_2\text{)-COOH}$, from the extract of a strip of the paper with *R_f* 0.65–0.75, and one spot at *R_f* 0.76, corresponding to $\text{C}_3\text{H}_7\text{-S-S-CH}_2\text{-CH(NH}_2\text{)-COOH}$, from the extract of a strip of the paper with *R_f* 0.9.

Accordingly, it can be concluded that the spot of *R_f* 0.12 is that of $\text{CH}_3\text{-S-S-CH}_3$, that of *R_f* 0.65–0.75 is $\text{CH}_3\text{-S-S-C}_2\text{H}_5$, $\text{C}_2\text{H}_5\text{-S-S-C}_2\text{H}_5$, $\text{CH}_3\text{-}$

$\text{S}-\text{S}-\text{C}_3\text{H}_7$ and $\text{C}_2\text{H}_5-\text{S}-\text{S}-\text{C}_3\text{H}_7$, and that of R_f 0.9 is only $\text{C}_3\text{H}_7-\text{S}-\text{S}-\text{C}_3\text{H}_7$.

Paper Chromatograph of Alliin Homologues in the Extract of Ipheion uniforum—From the above mentioned results, there seems to be no doubt that *Ipheion uniforum* contains three alliin homologues, namely, methyl-alliin, ethyl-alliin and propyl-alliin. To confirm this, paper chromatograph was run on these alliin homologues in the extract. As the result of this study, the spots of methyl-alliin, ethyl-alliin and propyl-alliin were observed in R_f 0.55, 0.15; 0.60, 0.15; and 0.65, 0.25 respectively.

II. Radish and Cabbage

From the new fact that there is the other plant than allium species such as *Ipheion uniforum* which contains alliin homologues in spite of the loss of alliinase, it will be necessary for us to make a study on the distribution of alliin homologues in the vegetable kingdom. So similar experiments were performed with the other plants than allium species. The experimental results show the similar facts with radish, cabbage *etc.* as with *Ipheion uniforum* (6). The following are the summary of the results.

Pyruvic Acid and Ammonia in the Extract of Radish—Pyruvic acid and ammonia were found in the extract of radish when the protein of garlic was added to it as shown in Table V.

TABLE V
Pyruvic Acid and Ammonia in the Extract of Radish

Material	Pyruvic acid (mg %)	Ammonia (mg %)
Extract of radish +protein of garlic	60	13
Extract of radish +boiled protein of garlic	0	0
Extract of radish	0	0

Production of Allithiamine Homologues by the Extract of Radish—Allithiamine homologue was detected by paper chromatograph in the mixture of the extract of radish with the protein of garlic and thiamine, when this mixture was heated at 60° for 30 minutes after the pH had been adjusted to 8.

Paper Chromatograph of Allicin Homologues—The methyl homologue of allicin was found only in radish as follows. One spot of allicin homologues at R_f 0.12 was found in the mixture of the extract of radish and the protein of garlic. As the results of the examinations of the alkyl-radicals of allicin homologues, only one spot at R_f 0.50, corresponding to $\text{CH}_3-\text{S}-\text{S}-\text{CH}_2-\text{CH}(\text{NH}_2)-\text{COOH}$ was observed in the extract of a strip of the paper with R_f 0.12.

Paper Chromatograph of Alliin Homologues—Two-dimentional paper chromatograph was carried on the alliin homologues and methyl homologue of

TABLE VI
Alliin Homologues in the Vegetable Kingdom

Material		Alliin homologues			
		Propyl-	Alyl-	Ethyl-	Methyl-
Liliaceae	<i>Allium sativum</i> Linnalus	+	###	—	+
	<i>Allium Bakeri</i> Regel	+	+	—	###
	<i>Allium tuberosum</i> Rottl	—	##	—	###
	<i>Allium victorialis</i> L.	—	##	—	+
	<i>Allium Cepa</i> L.	##	—	—	##
	<i>Allium fistulosum</i> L.	##	—	—	##
	<i>Allium Schoenoprasum</i> L.	##	—	—	##
	<i>Ipheion uniflorum</i> Rof.	+	—	+	##
	<i>Lilium lancifolium</i> Thunberg	—	—	—	—
Cruciferae	<i>Brassica oleracea</i> L.	—	—	—	##
	<i>Raphanus sativus</i> L.	—	—	—	##
	<i>Brassica pekinensis</i> Ruprecht	—	—	—	##
	<i>Brassica Rapa</i> L.	—	—	—	##
	<i>Brassica juncea</i> Cosson	—	—	—	+
	<i>Eutrema wasabi</i> Maximowicz	—	—	—	+
Compositae	<i>Lactuca sativa</i> L.	—	—	—	±
	<i>Chrysanthemum coronarium</i> L.	—	—	—	—
Umbelliferae	<i>Cryptotaenia japonica</i> Hasskare	—	—	—	±
	<i>Petroselinum sativum</i> Hoffmann	—	—	—	—
	<i>Daucus Carota</i> L.	—	—	—	—
Solanaceae	<i>Solanum tuberosum</i> L.	—	—	—	—
	<i>Lycopersicon esculentum</i> Mill	—	—	—	—
Rosaceae	<i>Malus asiatica</i> Nakai	—	—	—	—
	<i>Fragaria chiloensis</i> Duch	—	—	—	—
Ebenaceae	<i>Diospyros Kaki</i> Thunberg	—	—	—	—
Rutaceae	<i>Citrus Kinokuni Hortorum</i> ex Tanaka	—	—	—	—
Gramineae	<i>Oryza sativa</i> L.	—	—	—	—
	Rice-bran	—	—	—	—
	<i>Hordeum sativum</i> Jessen	—	—	—	—
Legminosae	<i>Glycine Max</i> Merrill	—	—	—	—
	<i>Arachis hypogaea</i> L.	—	—	—	—
	<i>Phaseolus vulgaris</i> L.	—	—	—	±

alliin was confirmed at R_f 0.55, 0.15.

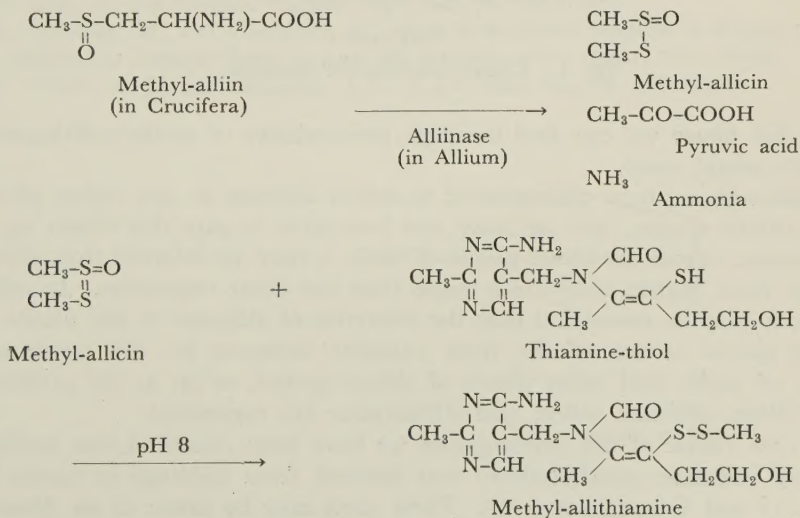
III. Distribution of Alliin Homologues in the Vegetable Kingdom

In order to examine the distribution of alliin homologues in the vegetable kingdom, the similar methods as in the cases of *Ipheion uniforum* and radish were applied for many plants. Table VI is the results of this study.

As shown in this table, alliin homologues are found mainly among the plants of Liliace and Crucifer. The plants of allium species contain alliin homologues most richly in quality and quantity. The methyl homologue of alliin is the most generally distributed in the vegetable kingdom and the ethyl-homologue of alliin is detected only in *Ipheion uniforum*. It must be noticed that propyl homologue of alliin is contained more widely in the plants of allium species rather than original alliin detected from garlic by Stoll *et al.* (7).

DISCUSSION

It must be evaluated that the nutritional significance of the plants of allium species has been elevated by the detection of the existence of alliin homologues in some other crucifera plants than allium species. For, methyl-allithiamine is produced from radish or cabbage by the aid of the action of garlic protein. The two following chemical formulas show the relations above mentioned.



We have clarified the facts that allithiamine is formed not only *in vitro* but also *in vivo* from allicin and thiamine contained in food and that the

places where allithiamine is formed *in vivo* are small intestine and blood plasma (8). On the other hand, it is well-known that alliinase reacts on alliin very promptly and converts it to allicin, pyruvic acid and ammonia. So it is no doubt that when we eat crucifera plants and raw allium plants simultaneously, methyl-alliin contained in crucifera plants will be decomposed to methyl-allicin by alliinase contained in allium species, in our stomachs and partly in mouths, and accordingly methyl-allithiamine will be formed by the chemical reaction between methyl-allicin and thiamine *in vivo*.

By the way, the properties of methyl-allithiamine are closely similar to that of allithiamine, particularly in its cell permeability. The following figure shows the hourly amount of thiamine found in urin in the first, second and third hour after an oral administration of 10 mg. of ordinary vitamin B₁ and of methyl-allithiamine respectively.

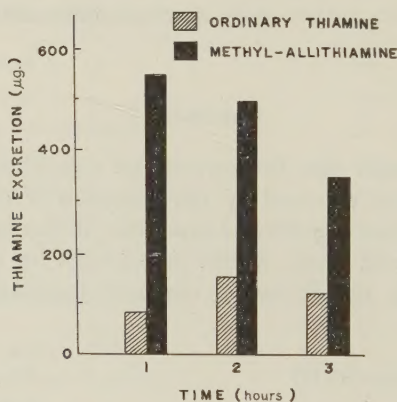


FIG. 1. Urinary excretion of thiamine.

From this figure we can find the high permeability of methyl-allithiamine from intestinal canal.

Although we have endeavoured to detect alliinase in any other plants than allium species, yet we have not been able to gain this object up to the present. From the above points of view, it may be inferred that allium species have higher nutritional value than the other vegetables. In other words, it may be concluded that the existence of alliinase in the plants of allium species is one of the most valuable elements for the nutritional worth of garlic and other plants of allium species, so far as the problems about alliin, alliinase, allicin and allithiamine are concerned.

In the course of this investigation we have been informed that methyl-cysteine sulfoxide (methyl-alliin) was isolated from cabbage or turnip by Morris and Thompson (9). These facts may be taken as an illustration of our studies.

SUMMARY

Starting from the discovery of allithiamine formed from the extract of garlic and thiamine, we have discovered the distributions of alliin homologues in any other plants than allium plants.

The alliin homologue is contained mainly in the plants of allium species and crucifera.

Alliinase detected by Stoll *et al.*, is generally contained in the plants of allium species, but not in the other plants than this species, even if alliin homologues is contained.

The nutritional significance of garlic has been discussed.

The authors express their hearty gratitude to Dr. U. Miura, Kyoto University, for encouragement, to Dr. T. Matsukawa and Dr. S. Yurugi, Research Laboratory, Takeda Pharmaceutical Industries, Ltd., for offer of help in various ways.

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EFFECT OF FATTY ACIDS ON THE PROTEOLYSIS OF PROTEINS

I. EFFECT OF C₆-C₁₄ FATTY ACIDS ON THE TRYPTIC DIGESTION OF OVALBUMIN

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(Received for publication, July 8, 1957)

It has been repeatedly demonstrated (1) that ovalbumin is easily digestible by enzymes after denaturation by heating, while, it is hardly digestible in the native state. The present author also reported (2) some results of her studies on the effects of heating on the tryptic digestibility of ovalbumin. The maximum digestion of egg white and egg albumin by trypsin was obtained by a heat treatment at 80° for 10 minutes (2, 3). When food protein is digested in the alimentary canal, it is usually accompanied with fatty acid. To clarify how the digestion of ovalbumin is affected by fatty acid, is interesting with respect to the physiology of digestion or enzymatic degradation of protein. The present study was designed to elucidate the effect of various fatty acids on the digestion of ovalbumin by trypsin.

EXPERIMENTALS AND RESULTS

I. Digestion Test

(A) Materials:

Crystalline Ovalbumin—Prepared by the modified method of Kekwick and Cannan.

(4). N-content 14.82 per cent.

Enzyme—Crystalline trypsin kindly supplied from Mochida Seiyaku Co. 1270 unit/mg.

Fatty Acids—Fatty acids used were *n*-caproic, *n*-caprylic, *n*-capric, *n*-lauric and *n*-myristic acids. All the acids were approximately 99 per cent pure.

(B) Experiments:

Preparation of Test Solution—An amount of 110 mg. of ovalbumin was taken into a 10 ml. conical flask and was dissolved with 2 ml. of water and 3 ml. of 0.3 *M* phosphate buffer (pH 7.8), followed by the addition of a definite amount of Na-salt of fatty acid. The final volume of the solution was made up to 10 ml. with water.

Enzyme Solution—The solution contained 5.5 mg. of crystalline trypsin in 1 ml. of water.

Denatured Ovalbumin Solution—The ovalbumin solutions were heated in a water bath (at 80° and 98°) for 10 minutes.

Tryptic Digestion—Digestion temperature: 37°, Digestion time: 1, 5, 10, 20 and 40 minutes, respectively. Three sets of 5 ml. of ovalbumin solution containing fatty acid were

treated as follows:

- a. control
- b. heated at 80° for 10 minutes.
- c. heated at 98° for 10 minutes.

Each of them was taken into 20 ml. test tube and kept at 37°. Thereupon, 1 ml. of enzyme solution was added to the test tube and mixed. At different time intervals, namely, after 1, 5, 10, 20 and 40 minutes' incubation, 1 ml. of the reaction mixture was taken out and poured into 10 ml. of 5 per cent trichloroacetic solution and the resulted precipitate was filtered off.

The optical density of the filtrate was estimated with a spectrophotometer at 280 m μ , and the digestion coefficient, (TCA soluble "tyrosin"/total "tyrosin" of ovalbumin $\times$ 100) was calculated. As for a blanc test, 0.83 ml. of the substrate solution, 0.17 ml. of the enzyme solution and 10 ml. of the trichloroacetic acid solution were mixed at the same time and filtered. The absorbance of this filtrate was subtracted from the above estimated absorbances.

(C) Results:

As shown in Table I, the proteolytic activity of trypsin towards denatured ovalbumin stopped almost completely after 40 minutes incubation under these conditions. The maximum degree of digestion by longer incubation time was approximately 80 per cent. As shown in Table I, C₆ fatty acid does not affect the digestion of ovalbumin by trypsin even at the concentration of 0.4 M; C₈ acid, however, accelerates the digestibility of ovalbumin at 0.1 M to almost the same degree as the heat denatured ovalbumin, and inhibits the digestion at higher concentrations. The longer the carbon chain of fatty acid, the stronger this effect appears, and C₁₂ acid has the effect for accelerating the digestion even at 0.015 M, and shows inhibition at 0.05 M. The C₁₄ acid also shows the same effects, at concentrations ranging from 0.0025 to 0.015 M.

II. Determination of Specific Rotation of Ovalbumin Solution

In order to recognize whether or not ovalbumin is denatured by the addition of fatty acid, the specific rotation of ovalbumin was determined,

TABLE I

Effect of Fatty Acid Added on the Digestion of Ovalbumin by Trypsin

(a) Control (without fatty acid)

Digestion time, (min.)	Treatment	Digestion coefficient (%)		
		Unheated	Heated	
			at 80°	at 98°
5		2.4	26.0	24.1
10		3.5	42.0	28.5
20		4.1	50.3	34.7
40		7.5	59.2	36.7

(b) Fatty acid added
C₆ *n*-caproic acid

Concentration (M)		Digestion coefficient (%)					
		0.1		0.2		0.3	
		Unheated	Heated at 80° at 98°	Unheated	Heated at 80° at 98°	Unheated	Heated at 80° at 98°
Digestion time, (min.)	Treatment						
5		3.2	34.8	29.5	—	2.7	—
10		5.8	34.3	31.0	—	6.5	—
20		7.2	43.3	37.5	—	8.2	—
40		8.1	50.3	41.3	—	7.6	—

C₈ *n*-caprylic acid

Concentration (M)		Digestion coefficient (%)					
		0.05		0.1		0.15	
		Unheated	Heated at 80° at 98°	Unheated	Heated at 80° at 90°	Unheated	Heated at 80° at 98°
Digestion time, (min.)	Treatment						
5		2.5	25.2	26.8	21.9	26.2	21.0
10		3.5	38.1	30.9	33.2	35.8	28.3
20		10.8	47.5	36.0	41.3	45.1	34.8
40		8.7	57.0	40.1	51.0	50.2	40.5

C₁₄ *n*-myristic acid

Digestion time, (min.)	Concentration (M)	Treatment	Digestion coefficient (%)							
			0.0025		0.005		0.015		0.05	
			Heated		Heated		Heated		Heated	
			Unheated	at 80°	Unheated	at 80°	Unheated	at 80°	Unheated	at 80°
5			11.6	39.4	15.5	39.7	37.1	50.7	13.4	8.4
10			16.7	49.6	19.9	54.0	51.1	62.5	19.9	18.1
20			26.8	59.9	22.8	61.8	57.1	70.4	24.2	23.8
40			18.9	66.9	23.3	64.9	60.4	76.9	26.3	26.5

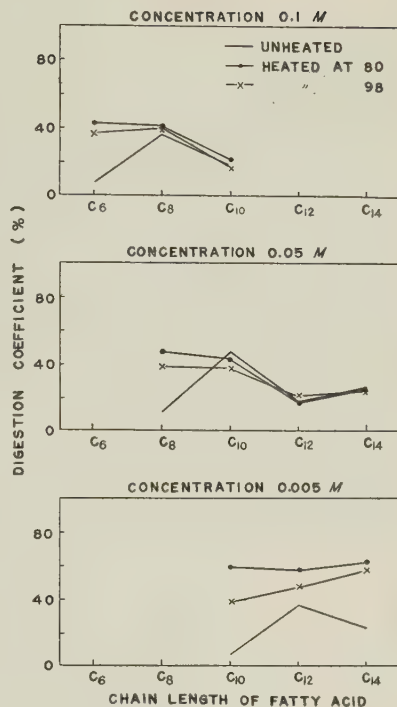


FIG. 1. Effect of fatty acid added at various concentrations on the digestion of ovalbumin by trypsin. Digestion time: 20 minutes.

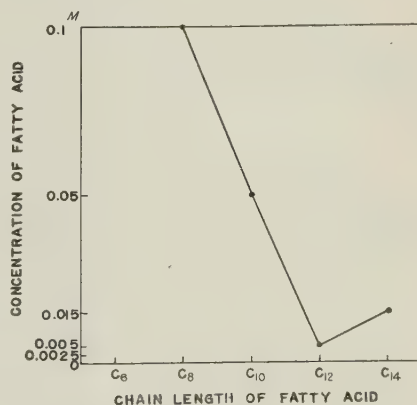


FIG. 2. Concentration of fatty acids at which the digestibility of native ovalbumin by trypsin exceeds 35 per cent. Digestion time: 20 minutes.

because the laevo-rotatory power of protein solution usually increases with its denaturation.

(A) *Samples:*

Native Crystalline Ovalbumin (Previously described)—1 per cent ovalbumin phosphate buffer solution (0.1 *M*, pH 7.8).

Fatty Acid Added Ovalbumin—One ml of 2 per cent ovalbumin phosphate buffer solution (0.2 *M*, pH 7.8) was added by proper quantities of fatty acid and 1 ml. of water to give final concentrations of 0.0025 *M*, 0.005 *M*, 0.015 *M* and 0.1 *M* respectively.

(B) *Experiments:*

The specific rotation of native ovalbumin (Sample a) was determined in a 5-cm. tube with Lippich's polarimeter at 25°. The fatty acid was added to ovalbumin solution (Sample b), the mixture was incubated 20 minutes at 37° followed by the centrifugation (12,000 r.p.m.) to remove the resulted turbidity. No detectable loss of nitrogen compounds by centrifugation was observed. The specific rotation was determined in the same way as above.

(C) *Results:*

It was shown that C_8 fatty acid did not cause any change in the optical rotation of ovalbumin solution even at the concentration of 0.05 *M*, but the rotation increased to laevo at 0.1 *M*. The longer fatty acids caused this

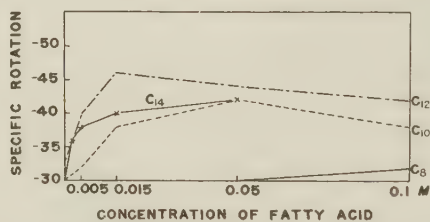


FIG. 3. Change in the optical rotation of ovalbumin by the addition of various fatty acids.

effect at lower concentrations, and C_{12} acid had the strongest effect at the concentration of 0.015 *M*.

DISCUSSION

The results obtained in the experiment I indicated that the addition of fatty acids to ovalbumin solution gave a change like the denaturation by heating, that is, an increase of digestibility by trypsin.

Concerning the effect of surface-active substances, it is reported by P.D. Boyer *et al.* (5) that denaturation of serum albumin is inhibited strongly by salts of fatty acids, especially by the acids whose carbon chain consists from 7 or 8 to 12 carbons. Also T. Sato recently reported that both the substrate (*i.e.*, hemoglobin, casein) and trypsin are denatured by sodium laurylsulfate (6). Considering these facts, it is apparent that ovalbumin is denatured by fatty acids and becomes more digestible by trypsin. This property of ovalbumin is entirely different from that reported on serum albumin (6). The optimum concentration of fatty acids at which tryptic

digestion of ovalbumin is distinctly promoted without heating differs from fatty acid to fatty acid. C_{12} acid had the strongest action among those tested and other fatty acids showed less effect. This is in accord with the fact that the denaturing action of alkylsulfate on the protein is most strong in C_{12} acid (7). The tryptic digestion of ovalbumin decreased above a certain concentration of added fatty acid, this might be explained by the assumption that fatty acids denaturate not only ovalbumin, but also trypsin itself as indicated in Sato's experiments. As the experiment II indicates, the optical rotation of ovalbumin solution is changeable with the addition of fatty acid, and the effect has almost the same tendency as on the tryptic digestion, that is, C_{12} acid has the strongest power at the lower concentration among the tested C_8 to C_{14} acids. It is generally believed that the change of optical rotation of the protein solution is caused by the denaturation, (of protein,) for instance by the unfolding of protein molecule (8). The results in the experiment I indicates that the denaturations by heating and by added fatty acids are probably the same kind of modification of proteins at least in a sense that both preparations are susceptible to tryptic digestion.

SUMMARY

1. C_8 to C_{14} -fatty acids promote the tryptic digestion of ovalbumin, but C_6 -fatty acid does not at concentrations 0.1–0.4 M .
2. Ovalbumin becomes susceptible to tryptic digestion by the addition of fatty acids nearly as much as by heat denaturation.
3. At higher concentration of fatty acids the tryptic digestion decreased for both heated and unheated ovalbumin.
4. The optimum concentrations of fatty acids for tryptic digestion of ovalbumin decrease with the increase of the carbon chain.
5. The laevo-rotatory power of ovalbumin solution increases by the addition of fatty acid. The effect differs from fatty acid to fatty acid.
6. The greatest change of optical rotation is obtained by C_{12} acid at 0.015 M concentrations, indicating that C_{12} acid is most effective in a modification of protein structure. This fits in with the observation by tryptic digestion.

The author wishes to express her sincere thanks to Prof. S. Akabori and Assist. Prof. B. Maruo for their kind guidance and encouragement throughout this work.

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A NEW ANTITUMOUR SUBSTANCE LENAMYCIN

ISOLATION AND PROPERTIES

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It was already established that trans-cinnamic acid amide (1) and ethoxy-ethene-1,2-dicarboamide (2) considered as the ethylated derivative of enol-oxalacetic acid diamide were isolated from the cultured broth filtrate of *Streptomyces* No. 902. Further studies on the fractionation of the antitumour cultured broth filtrate against HeLa (human cervix carcinoma cell in tissue culture) led to the particular metabolite, lenamycin, having antitumour activity at about 20 μ g. per milliliter of the tissue culture medium and having the properties of a specific organic acid amide. The isolation and its properties were herein reported.

The active agent in the cultured broth filtrate is markedly labile in weak alkaline reaction and moderately labile in weak acidic reaction. The

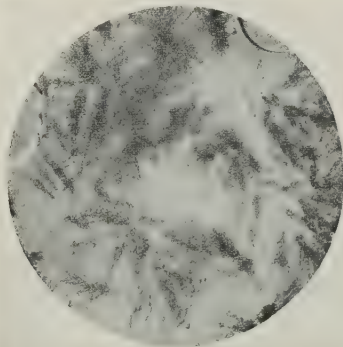


FIG. 1. Lenamycin in a crystalline status.

active agent obtained in the crystalline status (Fig. 1) by the isolation procedure described in the experimental section melts with decomposition at about 202–207° (d.p. 290–300°, unsharp, on aluminum block) after several times recrystallization from methanol and is optically inactive in methanol. The analytical data of the sample dried on P_2O_5 at 40° 8 hours under 10^{-1} mm. Hg show: C, 39.82–41.96 per cent; H, 4.42 per cent; N, 21.70–24.12 per cent and absence of sulfur, halogen or metal. The molecular weight

by Rast micro isothermal distillation method with water or methanol seems to fall in with between 80–160. Calculated for $C_4H_4N_2O_3$: C, 37.49 per cent; H, 3.15 per cent; N, 21.88 per cent and theoretical for molecular weight, 128. Calculated for $C_4H_4N_2O_2$: C, 42.86 per cent; H, 3.60 per cent; N, 24.99 per cent and theoretical for molecular weight, 112. The ultraviolet absorption spectrum in water shows a sole maximum at 216 $m\mu$ ($E_{1cm}^{1\%} = 817$) as in Fig. 2 and supports the suggestion that the compound is a typical

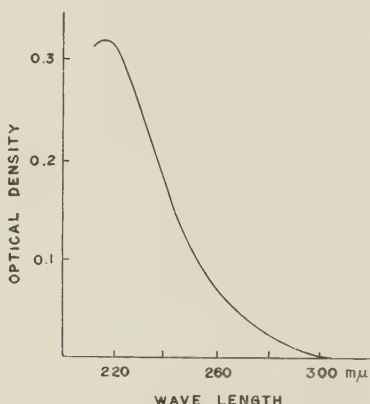


FIG. 2. The ultraviolet absorption spectrum of lenamycin in water.

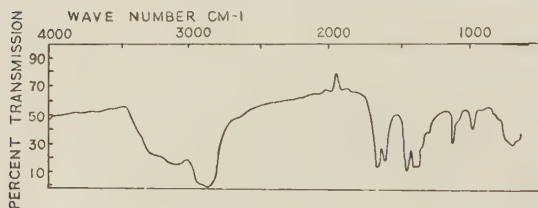


FIG. 3. The infrared absorption spectrum of lenamycin in nujol.

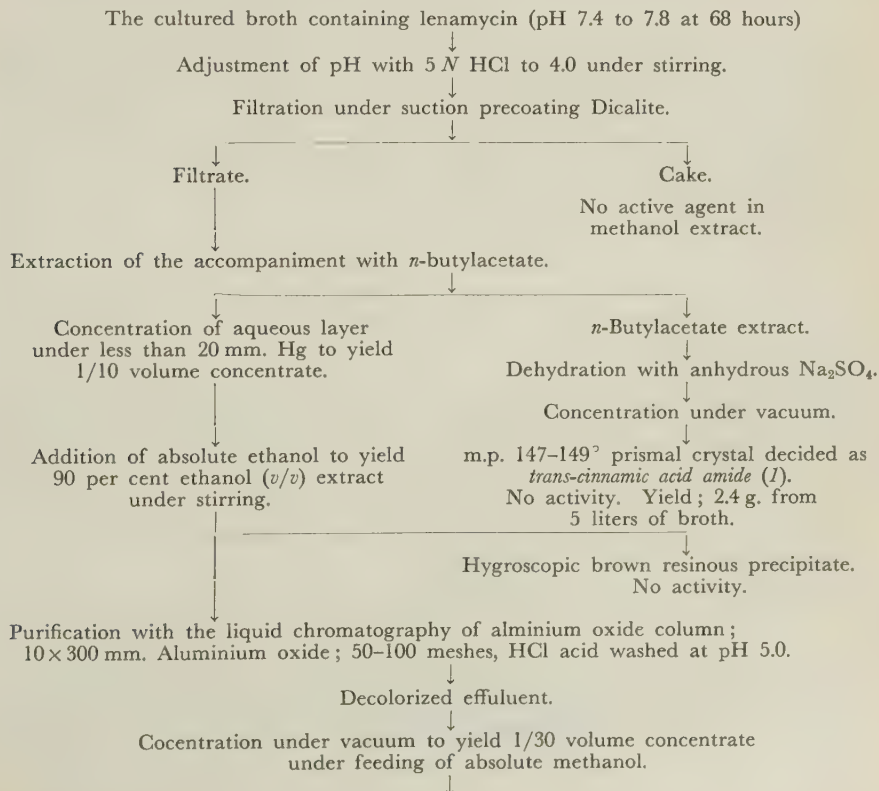
aliphatic one having an unsaturated bond conjugated with carbonyl group. The infrared absorption spectrum in nujol shows maxima at 3.07, 3.24, 6.00 and 7.12 microns assigning amide carbonyl group and at 6.00 with 6.20 microns assigning an unsaturated bond conjugated with carbonyl group. Further more, the absorption near at 8.90 microns seems to suggest the presence of etheric C-O bond, but it is doubtful from the ultraviolet absorption spectrum without bathochromic effect. The presence of nitril and diazo group are excluded by the infrared absorption spectrum. The presence of triple bond can not be ascertained from the infrared absorption spectrum, because the characteristic absorption band at about 4.5–5.0 microns may vanish under the symmetric substitution. Ninhydrin, biuret, anthrone and $FeCl_3$ reactions are negative. Elson-Morgan's and Sakaguchi's reactions are also

negative. The detection test for nitro or oxime group is negative. The alkaline degradation product by 1 *N* NaOH in a boiling water bath does not give any color reaction related to ethoxy-ethene-1,2-dicarboamide already reported (2) with FeCl₃ aqueous solution after the neutralization with 1 *N* HCl. On the other had, according to the above experimental evidences, it seems not yet to be elucidated whether ethoxy-ethene-1,2-dicarboamide already reported (2) is the true metabolite or the secondary product during the isolation procedure.

Thus it is interesting to consider that such a specific organic acid amide markedly inhibits the propagation of a carcinoma cell.

EXPERIMENTAL AND RESULTS

The Isolation of Lenamycin from the Cultured Broth Filtrate—The culture medium used is as following; glucose 2.5 per cent, molasse 0.5 per cent, soy bean meal 2.0 per cent, pea nut meal 1.0 per cent, meat extract 0.5 per cent, dried yeast 0.5 per cent, NaCl 0.25 per cent, CaCO₃ 0.2 per cent, pH 7.2; Sterilization, 120°, 5 minutes in an autoclave. The culture was performed by the conventional reciprocating shaker at 29° to 68 hours. The isolation procedure is as following.



PHYSIOLOGICAL CHEMISTRY OF THE HARD TISSUES

IV. RATE OF INCORPORATION OF DIETARY LABELED CALCIUM INTO GROWING BONE

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Isotopic tracer methods using Ca^{45} or P^{32} have been extensively used in the study of physiology and biochemistry of the calcified tissues and have made great contributions. However, because of physicochemical exchange of radioisotopes between body fluid and mineral crystals, it has been considered that they are of doubtful value as a quantitative indicator for the formation of the calcified tissues (1, 2).

By *in vitro* experiment, surface reaction of bone mineral has been intensely investigated not only qualitatively but also quantitatively (3, 4), but mechanisms responsible for radioactive calcium to be incorporated into calcified tissues *in vivo* are more complicated, both physicochemically and physiologically. Bone growth and reconstruction are active biological processes for the incorporation of radioactive calcium or phosphate. Moreover, physicochemical processes, *i.e.*, diffusion, ion exchange and recrystallization, also play important roles in the body. However, quantitative estimation on the role of any one of these mechanisms has not yet been made except in several special cases (4, 5).

In continuously growing incisor teeth of rats, it is possible to estimate quantitatively the ratio of physicochemical exchange to physiological accretion in the incorporation of radioactive calcium (6), but in bone tissues, biological reconstruction process puts the problem to further confusion.

The present study was attempted to obtain further informations on the difference in quantitative mechanism of isotope incorporation between the incisor teeth and bones, and it was found possible to estimate the amount of a fraction of radioactive calcium deposited into bones through growth.

METHOD

Administration of Calcium with a Stomach Tube—Growing albino rats were placed on a calcium-deficient diet with supplementary administration of calcium carbonate through a metal stomach tube connected to a glass syringe. This calcium-deficient diet was prepared by removing calcium carbonate from the diet described in a previous report (7). Since it was unavoidable that a small portion of calcium carbonate to be ingested would remain

in the syringe, amount of calcium really taken was calculated by subtracting remaining calcium from the original amount in the syringe.

When calcium carbonate labeled with Ca^{45} was administered, it was prepared from labeled calcium chloride and sodium carbonate as described in a previous report (7).

RESULTS

Administration of Calcium through a Stomach Tube—In order to examine the effect of supplying calcium through a stomach tube on general nutrition of the rat, young male and female rats, weighing 51 to 65 g. in male and 52 to 64 g. in female, were divided into two groups, which were placed on diets with or without calcium as described above. The rats on the normal control diet containing calcium ingested, on the average, 84 mg. of calcium daily during the experimental period. The rats on the diet without calcium were given 70 to 90 mg. of calcium as carbonate once a day with the stomach tube. Weekly gain in body weight of the rats are tabulated in

TABLE I

Weekly Growth Rate of Rats Maintained on Experimental Diets

Two groups of rats were placed on experimental diet with or without calcium. Rats on the calcium-deficient diet were given calcium carbonate with a stomach tube.

Administration of CaCO_3	Sex	No. of animals	Weekly body weight during experimental period (g.)							
			Initial	1	2	3	4	5	6	7
In the diet:	♂	3	59.1	82.2	102.2	130.8	152.9	165.3	180.0	205.0
Control group	♀	3	58.7	77.9	89.5	112.2	126.8	128.9	145.6	156.0
By the stomach tube:	♂	2	56.3	85.4	111.2	141.8	153.0	165.5	195.5	217.5
Experimental group	♂	3	59.5	86.0	109.3	127.7	144.0	157.0	167.0	179.7

Table I. No statistical difference between the two groups in body weight gain and in general appearance of animals is found and any abnormality in appearance was not observed in the group of the rats given calcium forcibly through a stomach tube. In other words, it may be inferred that calcium administered through a stomach tube can keep animals in a well-nourished condition.

Administration of Labeled Calcium through a Stomach Tube—After preliminary feeding with the diet containing normal amount of calcium for one week, young male albino rats of 90 to 110 days old, were given the calcium-deficient diet as described above with supplementary administration of calcium carbonate labeled with C^{45} . The rats were sacrificed by cutting their carotid artery 24, 48, and 72 hours after the ingestion of labeled calcium carbonate, and blood serum was collected from each rat. Specific activity of serum calcium and the amount of radioactive calcium in lower

TABLE II

Administration of Labeled Calcium with a Stomach Tube

Young male albino rats were given a calcium-deficient diet and calcium was given through a stomach tube as calcium carbonate labeled with Ca^{45} .

Time after CaCO_3 administration	Animal No.	Body weight	Labeled Ca in lower incisor*	Specific activity of serum Ca	Radioactivity in lower incisor*	Ingestion of labeled Ca
(hrs.)		(g.)	($\mu\text{g.}$)	(c.p.m./mg.)	(c.p.m.)	(mg.)
24	1	284	61		371	73.8
	2	271	78		474	80.6
	3	268	47		286	75.5
	4	259	79		481	83.2
	5	262	57		346	83.4
	6	288	68		414	83.4
	Mean	272	65		396	80.0
48	11	199	161	484	976	79.5
	13	217	117	404	716	81.0
	15	220	123	391	756	82.7
	17	228	150	424	915	79.5
	19	205	167	481	1018	85.3
	Mean	214	144	437	876	81.6
72	12	220	170	387	1030	74.7
	14	192	137	314	834	81.1
	16	198	182	426	1112	85.0
	18	198	220	590	1341	82.9
	20	204	214	562	1305	85.3
	22	230	203	536	1238	80.5
	Mean	207	188	469	1143	81.6

* Mean of observed values on two lower incisors.

incisor teeth were measured as in a previous report (7). The data are summarized in Table II.

DISCUSSION

Specific activity of the calcium incorporated into the incisor teeth is considered to be equal to that of serum calcium, when specific activity of serum calcium is kept at a constant level during the experimental period. In this case incorporation of radioactive calcium into calcified tissues by physicochemical exchange is considered to be active in the initial stage of the experiment and gradually approaches the maximum state as illustrated in a previous report of this series (6). When radioactive calcium is given by repeated injections to maintain specific activity of serum calcium at a constant level, incorporation of radioactive calcium by physicochemical exchange, in the initial stage of the incorporation-time curve, is estimated to be about 40 per cent of the overall radioactive calcium uptake by the lower incisor teeth of the rat (6).

In the experiment of Table II specific activity of serum calcium is indicated to have been maintained at a constant level from 48 till 72 hours after the administration of labeled calcium. In such a case total amount of deposited calcium can be computed from the equation (1) in a previous report of this series (6). In the experiment of Table II this calculation is

$$(1143-876) \text{ c.p.m./}453 \text{ c.p.m. per mg.} = 0.590 \text{ mg.}$$

Thus it is estimated that 0.590 mg. of calcium was deposited into one lower incisor tooth for 24 hours from 48th till 72nd hour of the experiment. The estimated value, 0.590 mg., agrees with the previously estimated value of 0.62 mg. for the amount of calcium incorporated into one lower incisor tooth daily to form new dental tissues, which was measured by the procedure based on quite different principles (6). Agreement of these two estimates increases the reliability of the estimated values and indicates that the increase of radioactive calcium in lower incisors caused by physicochemical exchange must be negligible in the period during 48th till 72nd hour of the experiment. Therefore, the accretion of calcium into one lower incisor tooth of the rat by new dental tissue formation is concluded to be about 0.6 mg. per day.

On the other hand dietary calcium labeled with Ca^{45} is incorporated into the incisor very slowly as indicated in Table II, *i.e.*, 0.065, 0.144, and 0.188 mg. of labeled calcium from one day's diet were found in one incisor tooth, respectively 24, 48, and 72 hours after the administration of labeled calcium through a stomach tube. According to Harrison and Harrison (8), absorption of Ca^{45} administered through a stomach tube reaches the maximum value within 24 hours of its administration. Therefore, the said results in Table II, *i.e.*, slow rate of the incorporation of labeled dietary calcium, could not be attributed to prolongation of the duration of labeled calcium absorption beyond 24 hours or more. Moreover, it has been indicated in a previous report (7) that even 12 days after the ingestion of labeled calcium, amount of the labeled dietary calcium found in the lower incisor was only 0.433 mg., as compared with 0.6 mg. of calcium being deposited daily by new tissue formation.

On the other hand the bone tissues take up more labeled dietary calcium than that expected from calcium retention (bone growth) as indicated in a previous report of this series (7) and by Harrison and Harrison (9). All of these observations are consistent with the idea that calcium absorbed from the gastrointestinal tract is deposited actively into bone tissues, not only through bone growth but also by physicochemical exchange and biological reconstruction process which are not directly related to the increase of skeletal calcium, and that the calcium is then liberated and again goes into circulating blood to be incorporated as a building material into newly forming mineral crystals of rat incisors.

It was reported previously that 155 mg. of labeled dietary calcium ingested for three consecutive days were found in an animal body, while balance measurement showed 109.5 mg. of calcium to be retained for the

said three days (7). Net increase of body calcium for the three days was 109.5 mg., but it certainly does not indicate that 109.5 mg. of labeled dietary calcium had been used in bone growth for the three days, because the rate of incorporation of labeled dietary calcium into bone minerals through the formation of new bone mineral must be as slow as the incorporation process of labeled dietary calcium in incisor. It is estimated that one lower incisor tooth of the rats took up $0.188 + 0.144 + 0.065 = 0.397$ mg. of dietary calcium from the diet ingested for three consecutive days, while $0.6 \times 3 = 1.8$ mg. of calcium was considered to be deposited into one lower incisor tooth through the accretion of new incisor tissue. Therefore, labeled dietary calcium taken up into the incisor for the three days corresponds to $0.397 \text{ mg.} / (0.6 \times 3) \text{ mg.} = 22$ per cent of the total amount of calcium deposited into the incisor through the formation of new dental minerals.

Since specific activity of calcium incorporated into bone tissues through the formation of new minerals should be equal to that of calcium incorporated into the incisors, it may be concluded that $(109.5 \times 0.22) \text{ mg.} / 155 \text{ mg.} = 15.6$ per cent of the labeled dietary calcium found in the animal body gives the estimated value of incorporated calcium coincident with the bone growth. In other words physicochemical exchange and biological reconstruction process deposited another 84.4 per cent of labeled calcium into the skeleton of rats.

A mode of the incorporation of radioactive calcium into bone tissues is considered to be different, as to quantitative mechanism, from that into continuously growing incisor teeth of a rat. In other words the physicochemical exchange and biological reconstruction process plays an important role in bone tissues, while deposition of radioactive calcium into incisor teeth of a rat is mainly due to its incorporation into new dental minerals as a building material.

SUMMARY

It was shown that rats placed on a calcium-deficient diet could be kept in a well-nourished condition when sufficient amount of calcium carbonate was given to the rats with a stomach tube. Rats, being fed as described above, were given one dose of calcium carbonate labeled with Ca^{45} in place of non-labeled calcium carbonate and sacrificed at intervals of 24 hours. The amount of labeled dietary calcium taken up by their incisor and specific activity of serum calcium were measured. The amount of calcium deposited into one lower incisor tooth of a rat through the formation of new incisor mineral has been estimated and the estimated value agrees with the previously measured value by the procedure based on quite different principles.

Physicochemical exchange seems to take only a little part in the fixation of labeled calcium by the incisor of the rat when labeled calcium is given orally as calcium carbonate. In such a condition, supposing that overall incorporation of radioactive calcium into the incisor is due to the formation of new minerals, the proportion of radioactive calcium deposited

through bone growth is estimated to be only 15.6 per cent of overall uptake of radioactive calcium by the skeleton. Another 84.4 per cent seems to be due to physicochemical exchange and biological reconstruction processes.

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THE INFLUENCE OF GROWTH PHASE ON ENZYMATIC ADAPTATION OF BACTERIA

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(Received for publication, October 26, 1957)

During investigation on the formation of pyrocatechase by bacteria, it was observed that the adaptability of the resting cells was greatly influenced by the growth phase of the cells. The adaptability of the cells was stronger in the stationary phase rather than in the log phase.

Similar observation was reported by Pinsky *et al.* (1) on formic hydrogenlyase and nitrate reductase and also by Arima *et al.* (2) on gentisase and *m*-hydroxybenzoic acid oxidase, so this phenomenon seems to exist widely.

The present report describes some experiments to clarify the conditions under which the adaptability of the cells in the log phase became as high as that of the cells in the stationary phase.

EXPERIMENTAL AND METHODS

Microorganism—The strain which was previously isolated to study the metabolism of pyrrolidone carboxylic acid (3) was used throughout the experiments. The taxonomical characteristics of the organism kindly examined by Komagata were as follows: rod (0.6–0.8 × 1.2–1.8 micron), motile possessing one to three flagella and gram negative. From these and previously reported observations (3) the organism was identified to be a *Pseudomonas*. The bacterium was grown in a medium containing 1 per cent meat extract, 1 per cent peptone, 0.3 per cent NaCl and pH 7.0 for 15 hours at 30° under aerobic condition. Two ml. of this culture medium were inoculated into 150 ml. of the new medium having the same compositions and incubated under the same conditions.

Pyrocatechase Activity and Growth Curve—The cells were harvested by centrifugation at appropriate time intervals, washed once with 0.2 per cent KCl and resuspended in the same solution. The pyrocatechase activity was determined manometrically (4) using resting cell suspension. The growth of the organism was estimated by a turbidimetric method and measuring the dry weight of the cells.

Extraction and Determination of Free Amino Acids within the Cells.—Free amino acids within the cells were determined by a modification of Gale's method (5). The cells were harvested from the growth medium, washed twice with 0.2 per cent KCl solution and resuspended in distilled water at a concentration of approximately 40 mg. dry weight per

10 ml. This suspension was then placed in the boiling water bath for 40 minutes. The suspension was centrifuged and precipitate was washed with small volume of distilled water. The supernatants were combined and evaporated to small volumes, and then extracted twice with 85 per cent ethanol. Alcohol soluble fraction was evaporated into small volume and reextracted with 85 per cent ethanol twice. Final ethanol soluble fraction were determined colorimetrically after development of color by ninhydrin according to the method of Moore *et al.* (6) using casein hydrolyzate as a standard. Two dimensional paper chromatography was carried out by ascending methods using following solvent system: butanol-acetic acid- H_2O (4:1:1) and phenol- H_2O (4:1). Glutamic acid, alanine and valine were assayed densitometrically according to Bull *et al.* (7) with one dimensional paper chromatograms developed by the butanol-acetic acid- H_2O (4:1:1) system.

RESULTS

The Relationship between Growth Phase and Pyrocatechase Adaptability.—The pyrocatechase activities of the cells at different growth phase were shown in Fig. 1. It was observed that the cells incubated for 3 hours, namely the

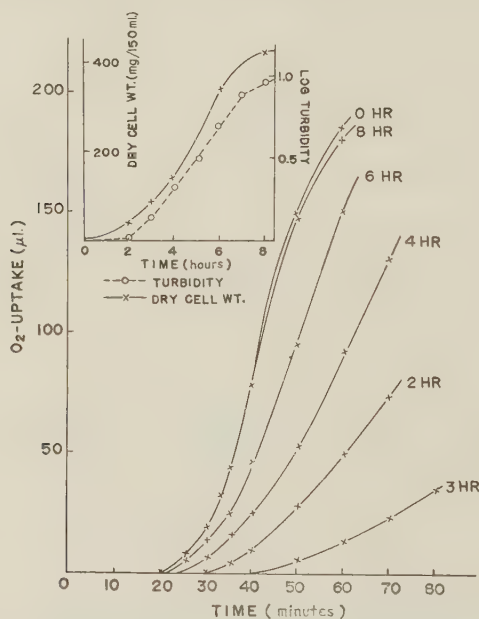


FIG. 1. Relationship between growth phase and pyrocatechase adaptability. Composition of reaction mixture of oxidation: cell suspension 0.5 ml., $M/5$ phosphate buffer (pH 7.0) 0.5 ml., $M/100$ catechol 0.2 ml. Data are expressed as microliters of oxygen taken up per mg. of dry weight of cells and corrected for endogenous respiration. The insert is the growth curve.

cells at the beginning of log phase (young cells), have the weakest adaptability among the cells from various other growth phases, in other words young cells showed a longer lag time in the adaptation pattern than

old cells. The cells at the stationary phase (old cells) showed the strongest adaptability, as indicated by its rapid adaptation.

Effect of Nitrogen Compounds on Adaptability of Young Cells—Since young cells showed a sluggish enzyme synthesis, it was assumed that the substances which were essential for enzyme formation were present in limiting quan-

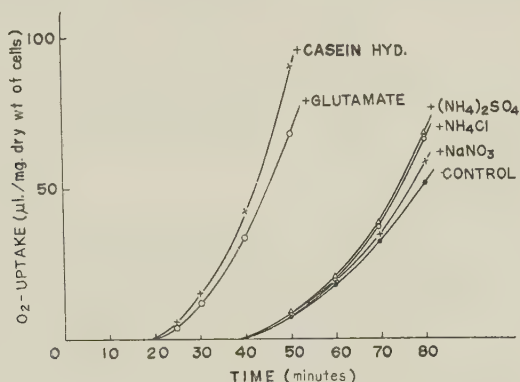


FIG. 2. Effect of nitrogen compounds on adaptability of young cells. Composition of the reaction mixture: cell suspension 0.5 ml., $M/5$ phosphate buffer (pH 7.0) 0.5 ml., $M/20$ catechol 0.1 ml., Added substances 0.1 ml. ($M/50$ Na-glutamate, 4 mg./ml. of casein hydrolyzate, $M/10$ NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$, NH_4Cl).

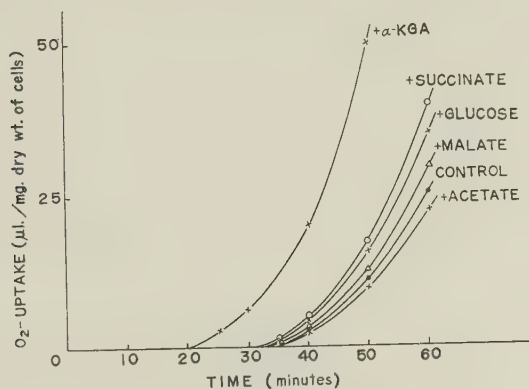


FIG. 3. Effect of non-nitrogenous compounds on adaptability of young cells. Composition of the reaction mixture: catechol $5\mu\text{M}$, added substances $5\mu\text{M}$, cell suspension 0.5 ml., $M/5$ phosphate (pH 7.0) 0.5 ml. Total volume 1.2 ml.

tities in the cells. In confirmation with this view, upon addition of casein hydrolyzate the rates of enzyme synthesis were markedly stimulated. The lag time was essentially identical with that of old cells under these conditions as shown in Fig. 2.

The same stimulation of enzyme synthesis was observed upon addition

TABLE I
Q_{O2} of Washed Young Cells and Old Cells for Various Substrates

Substrates	Glutamate	α -Ketoglutarate	Glucose	Succinate	Malate	Acetate	Valine
Young cells	110	10	20	70	60	20	3
Old cells	54	15	60	50		110	50

Q_{O_2} are expressed as microliters of oxygen take up per hour per mg. of dry weight of cell.

Composition of reaction mixture: cell suspension 0.5 ml., *M*/5 phosphate buffer (pH 7.0) 0.5 ml., *M*/10 substrate 0.1 ml.

of a single amino acid, glutamate, instead of casein hydrolyzate. While inorganic nitrogen compounds such as ammonium sulfate, ammonium chloride, sodium nitrate were not effective.

Neither yeast ribonucleic acid nor its acid or alkali hydrolyzate was effective.

Effect of Carbon-sources—The effects of glucose and several organic acids on the rates of enzyme formation were examined. As illustrated in Fig. 3, these substances had no effect on the adaptability of young cells, except α -ketoglutaric acid. Since young cells oxidized these substances as shown in Table I, the inability of glucose and several organic acids to stimulate enzyme synthesis could not be attributable to permeability barrier. The stimulation by α -ketoglutaric acid was almost comparable to that by glutamic acid at the same concentrations (Fig. 4).

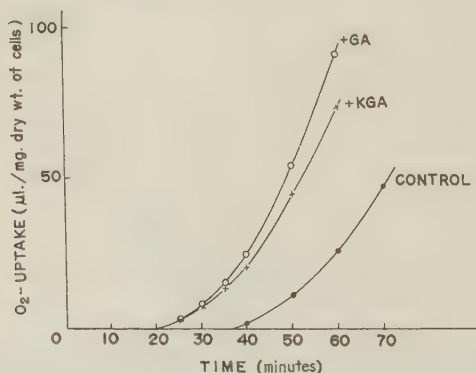


FIG. 4. Effect of α -ketoglutaric acid compared with that of glutamic acid on adaptability of young cells. Composition of the reaction mixture: catechol $5\mu\text{M}$, Na-glutamate or Na- α -ketoglutarate $2\mu\text{M}$, cell suspension 0.5 ml., *M*/5 phosphate buffer (pH 7.0) 0.5 ml., total volume 1.2 ml.

Effect of Shaking and Aeration—Washed young cells were suspended in *M*/20 phosphate buffer (pH 7.0) and were shaken or were bubbled with air for

2 hours at 30°. The aerated cells were then harvested by centrifugation and suspended in 0.2 per cent KCl solution and examined their adaptability. As shown in Fig. 5, the rate of enzyme synthesis was stimulated greatly after aeration. The same phenomenon was also observed in the adaptive oxidation of benzoate by this bacterium.

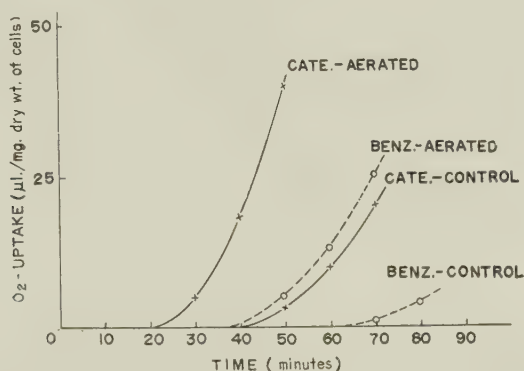


FIG. 5. Effect of aeration on adaptability of young cells. Composition of the reaction mixture: catechol or benzoate 2μM, cell suspension 0.5 ml., M/5 phosphate buffer 0.5 ml. Total volume 1.1 ml.

Free Amino Acids—As described above, amino acids were effective in restoring the adaptability of young cells. It was supposed that there was some differences in free amino acids within the cells between old and young cells. In order to examine this assumption total amounts of free amino acids of young and old cells were determined. As shown in Table II, total amounts of free amino acids in old cells were about twice of that in young cells. The aerated cells which had approximately the same adaptability with old cells, however, had almost the same amount of free amino acids as young cells, in other words, no appreciable increase or decrease in pool size of free amino acids was detected. Therefore, the difference in amino

TABLE II
Amounts of Free Amino Acids of Young and Old Cells

	Old cells	Young cell	Aerated young cells.
Total free amino acids (μg./100 mg. of dry weight of cells)	1170	647	586
Glutamic acid (μM/100 mg. of dry weight of cells)	1.5	0.62	
Alanine	0.49	0.26	
Valine	0.88	0.10	

acid pool size is not the only factor which determines the adaptability. In order to examine the composition of free amino acid fraction two-dimensional paper chromatography was carried out on both free amino acid fractions of young and old cells. Chromatograms showed the presence of 15 ninhydrin positive spots, 10 of which were identified from their R_f values to be glutamic acid, alanine, valine, aspartic acid, tyrosine, lysine, glycine, histidine and leucine or isoleucine. The paper-chromatographic pattern of young cells is almost identical with that of old cells. The major amino acids, glutamic acid, alanine and valine were determined quantitatively (Table II). Glutamic acid and alanine contents of old cells were approximately twice of those of young cells. Valine contents in young cells is approximately one-ninth of old cells. However, the rates of enzyme formation were not stimulated upon addition of valine.

DISCUSSION

It was observed that the pyrocatechase adaptability of young cells was stimulated approximately to the same level of old cells by following treatments, namely (a) amino acids or glutamic acid addition, (b) α -ketoglutaric acid addition and (c) treatment of young cells by aeration.

The same treatments of old cells did not effect on the lag time of old cells. The studies of free amino acids of yeast by Spiegelman *et al.* (8) indicate that free amino acids contributed to the formation of enzyme and a strong correlation existed between the enzyme synthesizing capacity and the availability of the internal amino acids.

In the present studies, it was found that there was little difference in their compositions of free amino acids between old and young cells but some differences existed in their amounts. From the results of total amino acids determination of young and aerated cells, the difference of their amounts did not effect on pyrocatechase adaptability.

From the studies on incorporation of radioactive carbon and phosphorus in *Torulopsis utilis*, Cowie *et al.* (9) indicated that there was a distinction between endogenous metabolic pools as essential intermediates for macromolecule synthesis and reservoir pools not on the main line of synthesis and furthermore the pool amino acids were not in the free form but were adsorbed to larger molecules and they were directly connected with the process of protein synthesis.

In the present experiments, young cells were in the actively growing state and the endogenous metabolic pools were probably directed to the synthesis of cell material, therefore pool amino acids could not be used for adaptive enzyme formation immediately.

The effect of α -ketoglutaric acid is interesting since the acid greatly stimulated the formation of pyrocatechase in the absence of added nitrogen compound. Further studies to clarify whether it may serve as a substrate for glutamic acid formation or not are in progress.

SUMMARY

Pyrocatechase adaptability of *Pseudomonas* was influenced markedly by the growth phase of the cells. The adaptability of the cells was weakest at the beginning of log phase, gradually increased and reached to the maximum level at stationary phase.

Supplement of glutamic acid to young cells caused an increase in adaptability to the level of the old cells.

Among non-nitrogenous compounds tested α -ketoglutaric acid was particularly effective in increasing adaptability of young cells. Strangely enough, just aeration of young cells in phosphate buffer was effective.

Free amino acids of young and old cells were determined. Usually old cells contained a larger amounts of free amino acids than young cells. The aeration technique, however, did not stimulate the accumulation of free amino acids within the cells. The relationship between free amino acid pool and adaptability was discussed.

The authors wish to express their many thanks to Prof. S. Akabori, Prof. S. Funahashi and Dr. B. Maruo for their kind guidance and many useful suggestions throughout the present work.

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INTERPRETATION OF THE ANTIVIRAL ACTIVITY OF VIRUSTATIC AGENTS IN A PHAGE/BACTERIUM SYSTEM

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The reproduction of virus in host cell is of necessity largely dependent on the physiological state of the host. This situation should be taken into account for the interpretation of virustatic activity. Employing a phage/bacterium system, much work has been done pertinent to the phage-static activity of various substances. Some of these works reasonably compared the phage-static and bacteriostatic activities of the agent tested (1, 2, 3). It is, however, noted that in these papers phage-static activity is determined by the one step growth experiment performed during a short time interval (not more than 1 hour), while bacteriostatic activity mostly determined by dilution technique, in which results are read after 1 day incubation. Thus, the comparison of the two activities is not carried out under the same experimental condition. Recently Bozeman *et al.* (4) tested the action of chloramphenicol on T1 bacteriophage by a carefully designed experiment in this regard.

In the present experiments phage-static and bacteriostatic activities are compared under an identical experimental conditions so far as possible. The final conclusion concerning the phage-static activity of an agent relative to the bacteriostatic activity is deduced from the correlation analysis of the data.

MATERIALS AND METHODS

Escherichia coli strain B and T2r bacteriophage were used as a test system. These were cultured in a nutrient broth of pH 7.4 at 37°. Bacteria-free stock phage suspensions titring 1×10^9 infectious units per ml. were prepared from lysates of soft agar cultures by repeated centrifugations.

The titration of virus content, measurement of adsorption, and one step growth experiments were performed essentially by the method outlined by Adams (5), except those modifications necessitated by the introduction of test substances. The virus and bacteria (logarithmic phase culture) were mixed together to give final concentrations of about 5×10^8 per ml., respectively. After an adsorption period of 2 min., the mixture was diluted 1:100 in antiphage serum. Five minutes were allowed for the serum inactivation of the free phage. Further two step dilutions (1:100 and 1:10,000) were made to the "first and second growth tubes." Thus, the final concentration of infected cells gave 5×10^4 and $.5 \times 10^2$ cells per ml. in the first and second growth tubes, respectively. All of these

procedures were carried out in a shaking rack in a water bath. Assays for the number of infectious centers were made from the both tubes at time intervals. In the present study all the assays were performed on the intracellular phages according to the cyanide method of Doermann (6).

The total bacterial cell number was traced nephelometrically and viable cells by the capillary tube method of Yanagita (7). Important was a point that the course of bacterial growth was traced most precisely during the period of initial 30 minutes, during which period virus multiplication completed in a parallel experiment. It was also taken into account to make the size of inoculum in bacterial growth experiment identical with the number of infected cells in the first growth tube, since the growth inhibitory activity of an agent was found to be largely affected by the size of inoculum in some instances (8).

Substances tested were sodium gentisate, hydrogen peroxide, penicillin, oxytetracycline and sodium cyanide. The gentisate was reported to exhibit selective toxicity on phage multiplication (2), while hydrogen peroxide (3) and penicillin (1) to be inactive on the phage reproduction. Oxytetracycline was chosen since it seemed to exert antiviral activity against certain animal viruses. A typical respiratory inhibitor, cyanide, also was used to demonstrate its antiphage activity.

RESULTS

Virucidal Activity—The direct virucidal action of five substances on T2

TABLE I

Virucidal Activity of Various Substances on Free T2 Phage Particles

Phages were subjected to contact with these substances for 30 minutes at 37° in nutrient media of pH 7.4.

Substance	Concentration	Per cent survival
Hydrogen peroxide	$1 \times 10^{-4} M^*$	112%
	$1 \times 10^{-3} \quad *$	84
	1×10^{-2}	50
Sodium gentisate	$1 \times 10^{-3} M$	113
	1×10^{-2}	99
	1×10^{-1}	12
Sodium cyanide	$1 \times 10^{-4} M$	101
	5×10^{-2}	96
Penicillin	10 Unit/ml.	73
	50	120
Oxytetracycline	0.3 $\mu g./ml.$	85
	1	116
	2	98
	10	173

* In these experiments the duration of contact with phages was 50 minutes.

phage was tested. After the incubation for 30 minutes at 37° of the mixtures of free phage suspension and graded concentrations of a test substance, the numbers of survival viruses were determined. As listed in Table I, any of these substances tested did not exert appreciable effect on the infective activity of free phage particles at the concentrations used in the following experiments.

Effect on Phage Multiplication—One step growth experiments of intracellular phage in the presence of graded concentrations of test substances were conducted. In these tests the agent was added in the “first and second growth tubes”. Taking notice of the major parameters in phage multiplication, *i.e.*, latent period, rise period and burst size, three types of growth inhibition were observed. These are graphically presented in Fig. 1. In case of gentisate (Fig. 1-a), only the burst size in phage multiplica-

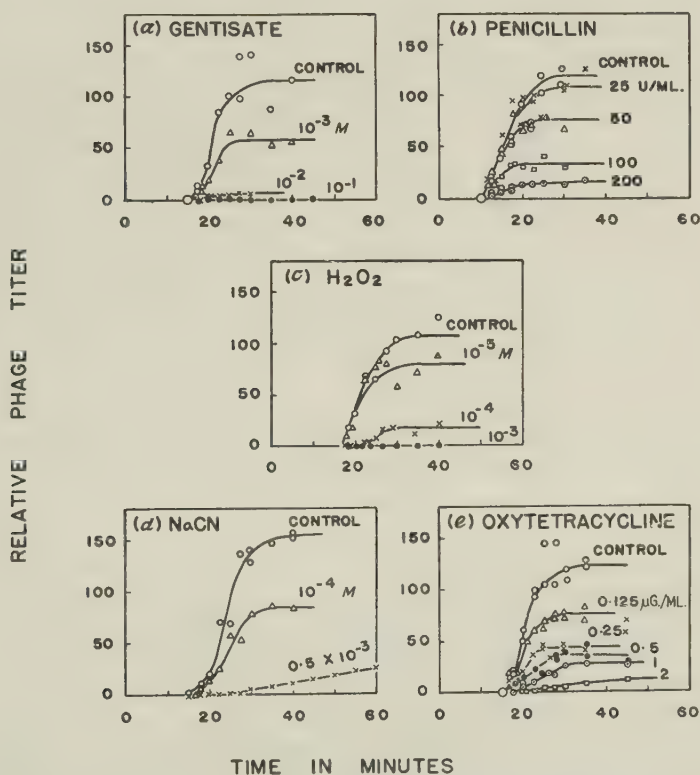


FIG. 1. Virustatic activity of various substances as shown by the effect on one step growth curve of intracellular phages. Input ratio phage/bacterium was about 0.1 in each experiment.

tion was minimized as concentration increased, leaving the latent period unaffected. Penicillin also was found to show such a type of activity (Fig.

1-b). Hydrogen peroxide, on the other hand, exerted its effect on the latent period as well as on the burst size, and not on the rise period (Fig. 1-c). Higher concentrations of this agent prolonged the latent period and retarded the level of burst size. The most complex type on growth inhibition was exerted by cyanide (Fig. 1-d). Higher concentrations of this substance prolonged the duration of latent and rise periods and suppressed burst size. Oxytetracycline appeared to show similar effect on the phage multiplication (Fig. 1-e).

Effect on Bacterial Growth—As already explained, attention was paid to make the experimental conditions in the antibacterial test identical with

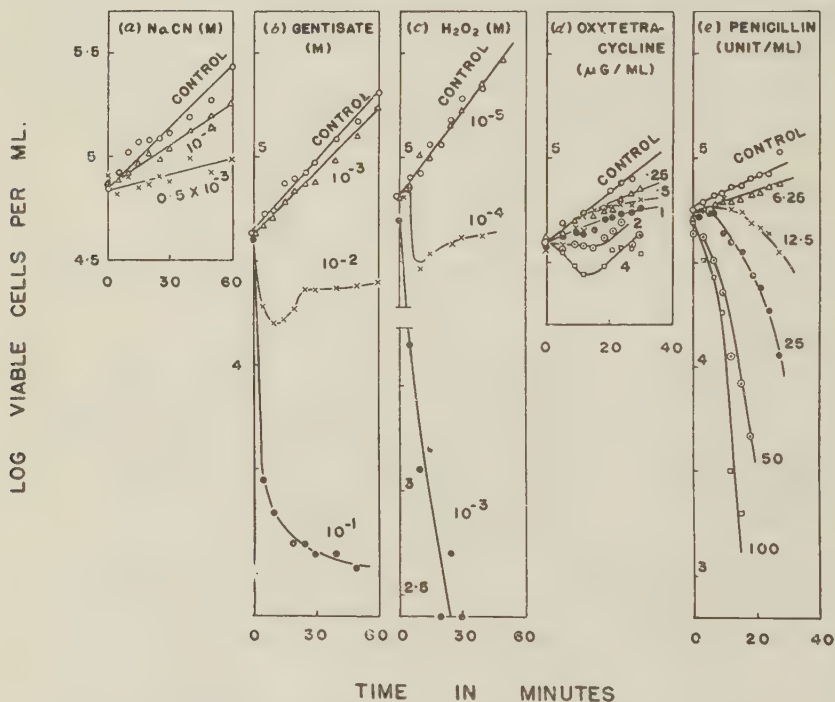


FIG. 2. Antibacterial action of various substances as shown by the effect on viable growth curve of *E. coli*.

those in the antiviral test. The age and size of inoculum and the duration of culture were the points strictly controlled. Under such experimental conditions, three types of growth inhibition were observed. Typical data are presented in Fig. 2. Cyanide (Fig. 2-a) inhibited the rate of growth statically simply through almost linear courses, while higher concentrations of gentisate and hydrogen peroxide (Fig. 2-b and c) inhibited the bacterial growth cidally instantly after the addition of the agent. Oxytetracycline (Fig. 2-d) may be classified into the latter group, although the initial bactericidal

activity was not so abrupt and striking as the other substances noted. Characteristic was the antibacterial action of penicillin (Fig. 2-e), with which the delayed appearance of bacteriolytic activity was demonstrated as reported earlier by Yanagita and Suzuki (9).

Comparison of Antiviral and Antibacterial Activities—In the present investigation an attempt was made to correlate the antiviral and antibacterial activities of an agent. Prerequisite for this purpose was the quantitative treatment of the data obtained in the preceding experiments. In case of phage growth inhibition, the suppression of burst size was taken into account, while in the antibacterial action the retardation of growth rate was taken as the measure of growth inhibitory activity. In the latter case, however, the quantitative measurement of activity was not necessarily easy, since in some instances the course of cultural development in the presence of a substance was not linear but much complicated. Therefore, the bacterial growth rate for the quantitative expression was arbitrarily determined to be the mean rate during the initial 15 minutes incubation; during this short period the formation of prophage in an infected bacterial cell can be expected. The degree of inhibition, I , of viral and bacterial growth was calculated by the following equation:

$$I = \left(1 - \frac{x}{x_0}\right) \times 100\%,$$

in which x and x_0 were the burst size (or bacterial growth rate) in the presence and absence of a certain concentration of a test substance, respectively. In this calculation, when the viable bacteria became less than the size of inoculum after 15 min. incubation, the growth rate was assumed to be zero.

Correlation analysis of the data thus calculated for viral and bacterial growth inhibitions was then conducted. In Fig. 3 each dot or cross represents the relationship between inhibitory activities on phage growth (ordinate) and bacterial growth (abscissa) caused by a certain concentration of a test substance. To make the precise analysis of the data as far as possible, the data obtained in the preceding experiments together with those obtained in repeated experiments also were plotted in the figures. Thus, we found three types of correlations. In Type I, represented by cyanide (dots) and hydrogen peroxide (crosses), each point plotted is nearly on the diagonal broken line. This fact shows that a parallel relationship exists between viral and bacterial growth inhibitions. In other words, the inhibitory activity on viral growth may solely depend upon the inhibition of the growth of host bacterium. As classified into Type II, oxytetracycline (dots) and gentisate (crosses) showed non-parallel relationship. All of the points plotted locate in an area between the ordinate and diagonal. This fact shows that these agents affect viral growth more strongly than bacterial growth, *i.e.*, slight inhibition of the growth of host cells resulted in a remarkable retardation on the yield of the virus. In contrast with the Type II, in Type III, as apparently evidenced by penicillin, data plotted locate in

an area between the diagonal and abscissa. In this instance the viral growth is less inhibited than the bacterial growth, *i.e.*, viruses are reproduced actively irrespective of the growth of hosts is largely affected by penicillin.

The action of oxytetracycline and penicillin, which are the respective representatives of the latter two types, are expressed in terms of dosage-response curves for viral and bacterial growth inhibitions (Fig. 4). The

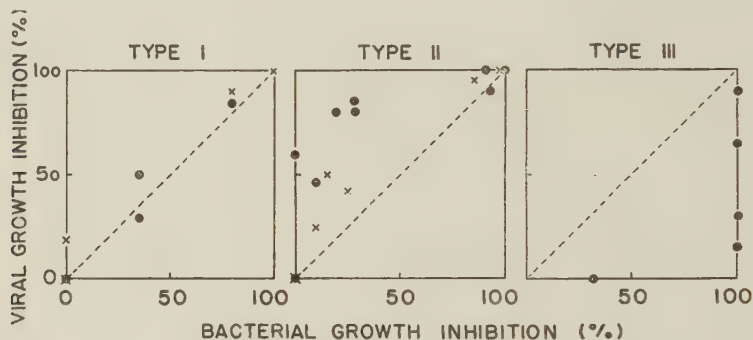


FIG. 3. Correlation analysis of antiviral and antibacterial actions of various substances. The relationship of inhibitory activities obtained in antiviral and antibacterial actions of a given concentration of an agent is plotted as a dot or cross. In Type I, dots and crosses represent data obtained for cyanide and hydrogen peroxide. In Type II, dots and crosses represent data obtained for oxytetracycline and gentisate. Data obtained for penicillin are plotted in Type III.

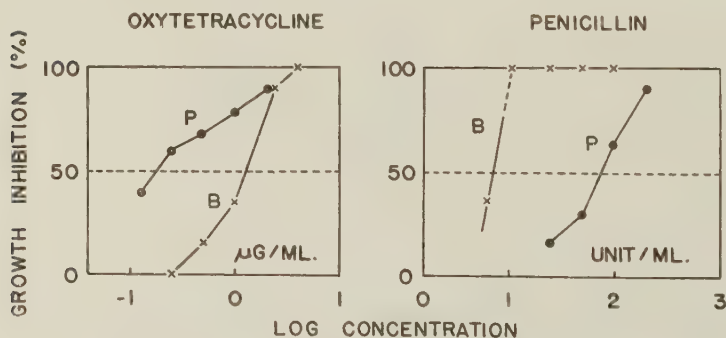


FIG. 4. Dosage-response curves showing the relationship between antiviral (P) and antibacterial (B) activities and the concentrations of oxytetracycline and penicillin.

relative positions of the dosage-response curves for both activities are in a reversed situation in these two instances. Activities of these agents against viral and bacterial growth can be quantitatively compared from these figures. Oxytetracycline concentration causing 50 per cent bacterial growth inhibition is found to be as 7.2 times as that of viral growth inhibition. The same ratio calculated for penicillin is shown to be 0.09.

DISCUSSION

In the interpretation of the action of antiviral substances, one should take into account for the action of the substance both on virus growth and on host growth. The comparison of these two activities should be conducted quantitatively under well controled identical experimental conditions as far as possible. The experiment designed without paying any of these precautions may sometimes lead us to the understanding of the phenomena far from real.

The present experiment was designed with special precautions in this regard. Quantitative analysis of the results obtained showed three types of mode of action of antiviral substances in a phage/bacterium system. Of the substances tested, hydrogen peroxide was known to be inactive on virus growth irrespective of its strong action on bacterial growth (3). Our experiment, however, showed that this substance was equally active both on virus and bacterial growth. Spizizen *et al.* (2) reported that sodium gentisate was exceedingly active on phage growth. They pointed out that the activity on virus growth was about 100 times as active as on bacterial growth. It was revealed, on the other hand, in the present experiment that although the gentisate was rather selectively toxic on virus growth, the selectivity was not so remarkable as they suggested. Interesting is the finding that oxytetracycline, which is believed to be active on animal viruses in some degree, was more selectively active on phage growth than on the growth of host bacterial cells.

It may be obvious that the results thus obtained in phage/bacterium system cannot necessarily be applicable to animal viruses. If it is possible to conduct such a sort of experiment using a system of animal virus/host (intact animal or tissue culture), it may afford some sense in the evaluation of antiviral substances.

SUMMARY

Employing T2 phage/*Escherichia coli* strain B system, the mode of several antiviral substances on virus multiplication was investigated. The actions of these substances on virus and bacterial growth were compared quantitatively under identical experimental conditions as far as possible.

Correlation analysis of the results obtained revealed three typical types of mode of action of antiviral substances. In Type I, substances (cyanide and hydrogen peroxide) were found to exert parallel activities on virus and bacterial growth. On the other hand, Type II substances, such as gentisate and oxytetracycline, showed selective toxicity on virus growth, whereas Type III substance, represented by penicillin, showed selective activity on bacterial growth.

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CRYSTALLINE BACTERIAL PROTEINASE

I. PREPARATION OF CRYSTALLINE PROTEINASE OF *BAC. SUBTILIS*

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Crystallization of proteinase from the culture medium of a strain of *Bacillus subtilis*, *Bacillus amyloliquefacience* (1), was reported by J. Fukumoto and H. Negoro in 1951 (2), but their method appeared to be not reproducible. Güntelberg and Ottesen reported a preparation of crystalline proteinase, a plakalbumin-forming enzyme (3), from the medium of *B. subtilis* in 1952 (4). Proteinase of *Bacillus Natto* which is thought to belong to *B. subtilis*, was also crystallized by Miyake *et al.* in 1954 (5). An attempt has been made to obtain crystalline proteinase in good yield from several selected strains of *B. subtilis* which are denoted as *B. sub. P, R, H, N*, and *N'* in our laboratory (6), but it was found to be very difficult or almost impossible to get it from the medium of the above strains by the combination of routine procedures such as salting out, organic solvent precipitation, or adsorption on mineral adsorbents.

Recently, a few kinds of ion exchange resin have been found to be useful for the purification of several enzymes. Especially a polyacrylic acid cation exchange resin, Amberlite IRC-50, has been used as adsorbent of bacterial pectinase (7), cytochrome c (8), ribonuclease (9), lysozyme (10), and chymotrypsinogen (11). However, this resin hardly adsorbed the proteinase of the above bacteria from the solutions under any conditions except at very low pH and at low concentration of salts. After many unsuccessful attempts employing numerous resins, it was found that the enzyme could be adsorbed on the polyphenol-phenoxy-acetic acid resin specially prepared by Scientific Research Institute Ltd., and crystalline proteinase of *B. sub. H, R, N*, and *N'* was easily obtained in good yield using this resin (6, 12). This crystalline proteinase has been used for the study on "denaturation and inactivation of enzyme proteins" (13, 14). After the above investigation, another cation exchange resin, Duolite C-10, was found more effective for the above procedures, *i.e.* it has better adsorption capacity and adsorbed proteinase can be eluted in higher concentration. In this paper is presented the improved

* Present adress; Nagase & Co., Ltd., Amagasaki Factory, Amagasaki, Japan.

method for purification and crystallization of proteinase of *B. subtilis* using the above resin.

MATERIALS

Enzyme—A commercial proteinase preparation, “Bioprase”, supplied from the Nagase & Co., Ltd. was mainly used. The preparation was obtained from submerged culture solution of *B. sub. N'* by precipitation with ammonium sulfate at 0.6 saturation after separating amylase by the raw starch adsorption method. Other proteinase preparations prepared by the ammonium sulfate precipitation from culture solutions (culture medium E, F, A-4 or A-2 added with 0.5 per cent casein in the previous report (6) was useful) of *B. sub. H, P, R*, and *N* and also a strain of *B. Natto* were successfully used.

Resin—Duolite C-10, a highly porous phenolic resin having ω -sulfonic acid as a functional group was used. One litre of the commercially obtained H-form resin was converted to Na-form by suspending in about 3 litres of 1 *N* sodium hydroxide and washed twice with about 10 litres of tap water by decantation. The suspended resin was crushed in a ball mill or Waring blender, and larger particles were removed by passing the suspension through a 60 mesh sieve by the aid of occasional jet of tap water on the sieve. The resin on the sieve was resuspended in water and further ground. From the resin passing through the sieve, fine particles were removed at first 30 minutes' decantation, then using a 150 mesh sieve. The 60-150 mesh resin was stored by suspending in water.

METHODS

Assay for Proteinase Activity—Hagihara's method (6), based on the methods of Anson (15) and Kunitz (16), was further modified as follows*: One millilitre of enzyme solution, which was suitably diluted with 0.02 *M* sodium chloride (or sodium phosphate buffer), was mixed at 30° with 5 ml. of 1.2 per cent casein solution containing 0.03 *M* ammonium phosphate buffer, pH 7.5 or pH 10.0. After 10 minutes' reaction at 30°, 5 ml. of protein precipitation reagent (containing 0.11 *M* CCl_3COOH , 0.22 *M* CH_3COONa , and 0.33 *M* CH_3COOH —Reagent A in Method for Enzyme Investigation—by which better relation between activity and measured value was obtained (6)) was added to the reaction mixture and the resulting suspension was filtered through a filter paper used for quantitative analysis** after standing at 30° for about 30 minutes. The optical density of the filtrate was read at 275 μ . The readings were corrected for the value of blanks, in which enzyme solutions were mixed with the precipitation reagent before the addition of substrate.

One unit of the proteinase activity was defined as the activity of enzyme which liberates the digestion product not precipitated by the above reagent and giving the extinction at 275 μ equivalent to 1 μ g. of tyrosine in 1 minute' reaction at 30°. The unit was corrected by a standard activity curve and represented by $[\text{PU}]_{\mu\text{g. tyr.}}^{\text{cas. 275}}$. A better parallel relation was obtained between enzyme activity (concentration) and measured value

* The detailed procedure was described by B. Hagihara in *Proteolytic Enzyme* in “Method for Enzyme Investigation” edited by S. Akabori, Asakura-Shoten, Tokyo, Vol. 2, p. 237-246 (1956).

** Some substance showing absorption at 278 μ was eluted with the trichloroacetic acid solution from ordinary filter papers.

by the use of the above precipitation reagent than by the usual rather concentrated trichloroacetic acid alone (Fig. 1).

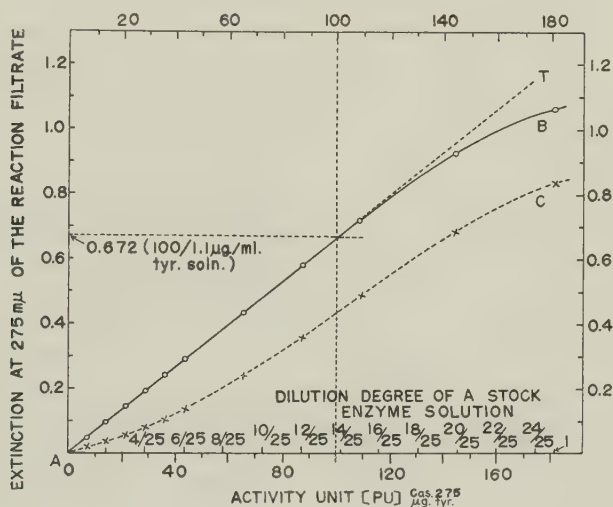


FIG. 1. Standard curve for assaying bacterial proteinase by casein-275 $m\mu$ method. Stock enzyme solution: about 80 $\mu\text{g.}/\text{ml.}$ solution of BPN'. Reaction: 1 ml. of enzyme solution and 5 ml. of 1.2 per cent casein solution buffered at pH 7.5, at 30° for 10 minutes. Precipitation reagent: 5 ml., 0.33 M CH_3COOH , 0.22 M CH_3COONa , 0.11 M CCl_3COOH mixture for Curve AB, 0.44 M CCl_3COOH for Curve AC. Activity unit: $\mu\text{g.}$ tyrosine equivalent per ml. enzyme (see the text), was calculated as follows, after correction by tangent line AT:

$$\begin{aligned}
 [\text{PU}]_{\mu\text{g. tyr.}}^{\text{cas. 275}} &= \frac{\text{OD measured}}{\text{OD of } \mu\text{g./ml. tyrosine}} \times \frac{\text{volume (ml.)}}{\text{reaction time (min.)}} \\
 &= \frac{\text{OD measured}}{\text{OD of } \mu\text{g./ml. tyr.}} \times \frac{11}{10} \\
 &= \frac{1.1}{\text{OD of } \mu\text{g./ml. tyr.}} \times \text{OD measured} \\
 &= \frac{\text{OD}}{0.00672}
 \end{aligned}$$

Preparation of Resin Column—About 500 ml. of the ground resin (70–150 mesh at swelled Na-form) was suspended in 2 litres of 1 N sodium hydroxide at room temperature for about 2 hours, transferred to a large glass filter, and then washed by passing 10 litres of tap water. The resin was converted to H-form by passing about 2 litres of 1 N hydrochloric acid and washed again with 10 litres of water. About 1 litre of sodium phosphate buffer solution (pH 6.5) containing 0.2 N sodium ion (hereafter abbreviated as 0.2 N Na^+ buffer) was passed through the washed resin. The resin was suspended in 1 litre of the same buffer and pH of the suspension was brought to 6.7 with 12 N sodium hydroxide. As the pH fell rapidly, further sodium hydroxide was added every several minutes to maintain the suspension at pH 6.6 while stirring it for 1 hour, and then the suspension was allowed

to stand overnight. The pH fell to 6.5 and became constant. The resin was transferred to a glass filter and washed by passing about 3 litres of the buffer (pH 6.5) slowly. The buffered resin suspension was introduced into a column, 3 cm. in diameter and about 80 cm. in length, held strictly vertical until the settled resin bed became 60 cm. in height.

Preparation of the Enzyme Solution for the Resin Treatment—About 100 g. of the commercial bacterial proteinase preparation, Biopraxe, was well suspended in 800 ml. of water and adjusted to pH 6.5 with ammonia*. After standing at room temperature for 2 hours, insoluble material (mainly Celite) in the suspension was removed by filtration through a Büchner funnel and residue on the funnel was washed with about 300 ml. of water. Thus, about 1 litre of dark solution showing proteolytic activity of about 3×10^5 unit at pH 10 was obtained. The enzyme solution was divided into 5 cellophane bags and dialyzed at first against running tap water for 3 hours, and then against 10 litres of 0.05 *N* Na-phosphate buffer (pH 6.5) overnight. Any precipitate that appeared during the dialysis was removed by filtration with a small amount of Hyflo Super Cel.

In the case of culture solution of *B. subtilis*, the enzyme was precipitated with 400 g. of ammonium sulfate per litre of solution and separated from the solution by filtration using Hyflo Super Cel. The cake on the funnel was washed with the ammonium sulfate solution and pressed between two large sheets of filter paper in order to remove the ammonium sulfate solution. The drained cake was treated as above.

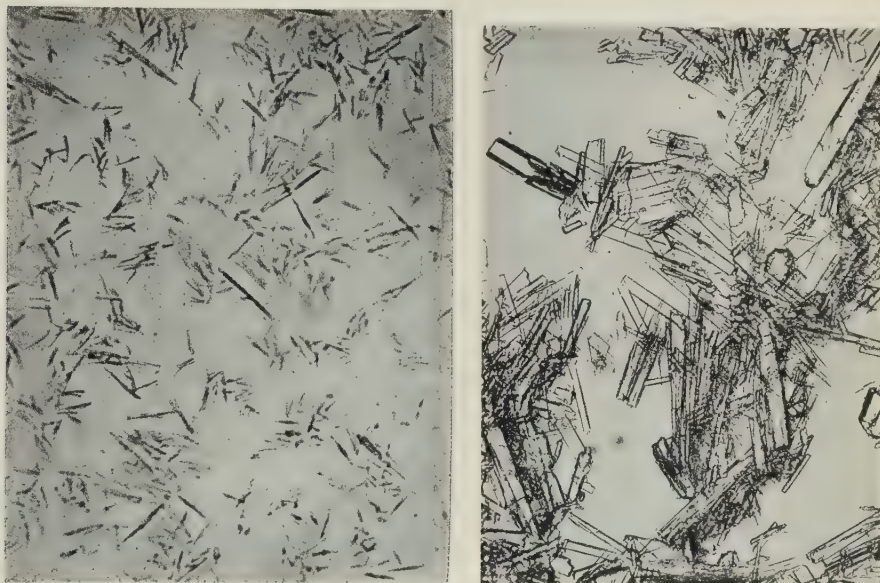
Column Treatment—About 1.5 litre of the dialyzed enzyme solution having about 2×10^5 proteolytic unit was passed through the above Duolite C-10 column at the rate of 3–5 ml. per minute at 5–10°. The column adsorbing the enzyme was washed with about 5 litres of tap water, 0.5 litre of 20 per cent acetone, 1 litre of distilled water, and finally 1 litre of 0.2 *N* Na⁺ buffer of pH 7.0. The enzyme was then eluted from the column by passing about 2 litres of a solution containing 0.1 *M* NH₄OH, 0.1 *M* Na₂HPO₄, and 0.5 *M* NaCl, at the same rate as the adsorption process. The effluent was easily divided into 4 parts by their color and protein precipitation test with trichloroacetic acid: Fraction I—about 100 ml. of colorless solution of pH 6–7 containing none or very small amount of protein; fraction II—about 500 ml. of colorless solution of pH 6–7 containing a very large amount of protein (mainly proteinase); fraction III—about 200 ml. of slightly yellow or brown solution of pH 7–9 containing a rather large amount of protein (also mainly proteinase); and fraction IV—dark brown solution of pH 9–10 containing slight amount of protein. In the above Fraction II, more than 60 per cent of the adsorbed proteinase was present.

Crystallization—Crystallization of the proteinase from the above Fraction II was carried out similarly as reported before (6). The enzyme was precipitated by the addition of 40 g. ammonium sulfate per 100 ml. and the precipitate was collected on a small Büchner funnel with the aid of 5 g. of Hyflo Super Cel per 100 ml. The precipitate was washed with ammonium sulfate (0.4 g./ml.) solution of pH 7.0 and the washings were strongly sucked out. About 20 ml. of 80 per cent acetone, followed by 100 ml. of 75 per cent acetone kept at 40°, was passed through the precipitate on the funnel with gentle suction in order to remove the ammonium sulfate.

The washed precipitate was suspended in 50 ml. of 0.02 *M* sodium acetate for 10 minutes, then Cel was removed by filtration and washed with 30 ml. of the above solution. From the combined filtrate and washings, the enzyme was precipitated by the addition of 2.5 volumes of 96 per cent acetone at 5–10° and collected by centrifugation at $3,000 \times g$.

* The amount of the enzyme preparation should be changed according to its proteolytic activity.

Acetone was removed from the precipitate collected in a 50 ml. centrifuge tube by blowing air. When the precipitate had almost dissolved, the suspension was maintained at about 15° for 10 minutes, then a slight amount of insoluble matter, if such formed, was removed by centrifugation. To the concentrated proteinase solution (containing more than 10 per cent of the enzyme) a few drop of acetone was added and the resulting precipitate was dissolved by shaking the solution after the tube was stoppered tightly. The same procedure was repeated until no more precipitate dissolved. When the resulting slightly turbid solution was kept at 15° , needle crystals appeared in several minutes and rapidly increased (Fig. 2, A). The suspension was kept at 15° for 30 minutes, then at 10° for 2 hours, and



A. Crude crystals ($\times 600$)

B. Good crystals ($\times 200$)

FIG. 2. Crystalline proteinase of *B. subtilis* *N'*.

finally in an ice box at $4-7^{\circ}$ for 2 or 3 days. Since the large amount of crystals made the suspension a hard mass, it was stirred by a glass rod and centrifuged at $3,000\times g$. The crystals were suspended in 3 volumes of 55 per cent acetone containing $0.02 M$ sodium acetate at $2-5^{\circ}$ for 10 minutes, then centrifuged.

The washed crystals were dissolved in minimum amount of $0.02 M$ sodium acetate and recrystallized similarly as above. In this case, the appearance of the crystals was usually very slow and they formed fairly large plates after the suspension was stood at about 5° for several days with occasional addition of a few drops of acetone (Fig. 2, B).

RESULTS AND DISCUSSION

Adsorption of Proteinase and Impurities on Resins—The proteinase of *B. sub. P, H, R, N*, and *N'* was not adsorbed on Amberlite IRC-50 or XE 64 under the conditions used for the adsorption or chromatography of cytochrome c (8, 15-19), ribonuclease (9), lysozyme (10), and chymotrypsinogen (11).

Boardman and Partridge (17) reported that a protein, cytochrome c, was more strongly adsorbed on the above resin from a more diluted salt (cation) solution. This was also true in the case of bacterial proteinase and the solution of a very low salt concentration, *e.g.* below 0.02 *M* sodium ion, but the amount adsorbed was very slight. Therefore, this resin was not suitable for purification of the enzyme.

The low cross-linking polyphenol-phenoxyacetic acid cation exchange resin specially prepared by the Scientific Reserch Institute, Tokyo, Japan, showed much better adsorption than Amberlite IRC-50 and was used for the purification and crystallization of the enzyme (6). However, this resin is not commercially available and its adsorption capacity varied considerably from lot to lot.

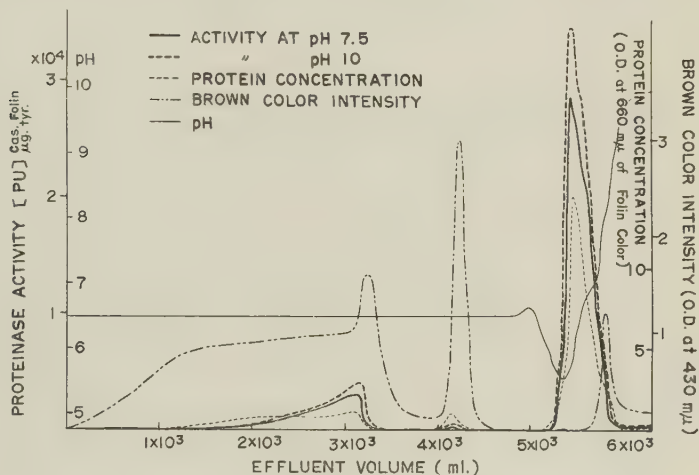
The present resin, Duolite C-10, has shown several times as large adsorption capacity of the enzyme as the best preparation of the Scientific Reserch resin. These two resins behaved similarly as regards to adsorption of the enzyme and impurities, but very differently from Amberlite IRC-50. Though the resin, Duolite C-10, and also the Scientific Reserch resin showed a higher adsorption at a lower pH as the Amberlite IRC-50, their adsorption capacity was slightly affected by the salt concentration, contrary to the case in the latter resin.

The present resin adsorbed a fairly large amount of coloring matter, melanine-like substances of dark brown color, contained in the crude enzyme solution, and this adsorption was much affected by the pH and salt concentration, and also by the presence of organic solvents. The adsorption was larger at a lower pH and a higher salt concentration, but became much smaller in the presence of 10–40 per cent acetone or ethanol. On the contrary, adsorption of the enzyme was only slightly affected by them. Among many impure proteins contained in the source material, amylase was partially adsorbed together with the proteinase, but the adsorption was much weaker than the latter.

Resin Column Treatment—Considering the above facts and stability of the enzyme at the same time, the procedure described in the Methods was adopted. When the dialyzed enzyme solution of pH 6.5 was passed through the column of the resin buffered at pH 6.5, the proteinase was almost completely adsorbed on the column and almost all of amylase and a larger part of the coloring matter passed through. It was not impossible to adsorb proteinase completely by passing a smaller amount of the enzyme solution but ratio of impurity and proteinase adsorbed on the column increased by it. Therefore, it was better to charge the amount of the enzyme solution to about 10 per cent in excess. By the washing with water, pH of the effluent became slightly higher and a large amount of coloring matter was eluted together with a small amount of proteinase. By further washing with 20 per cent acetone, the coloring matter was eluted but almost no proteinase was lost.

When the alkaline solution containing high concentration of salt was

passed through the column, the resin became dark in color from the top of the column by the change of the resin to alkaline form. Since this alkaline form of the resin had no adsorption power for the enzyme and also slight for the coloring matter, they were eluted down from this part and adsorbed again on the lower part of the resin which was still in neutral form. In this case, the above readsorption of the coloring matter was stronger than that of proteinase because the eluting solution contained an extremely high con-



Concentration of protein (Optical density of Folin color —test soln: Na_2CO_3 : Reagent; 1:5:1— of precipitated substance with trichloro acetic acid)

Proteinase activity at pH 10 by Casein -275 $\text{m}\mu$ method.

Brown color (Optical density at 430 $\text{m}\mu$)

FIG. 3. Adsorption and elution operation of bacterial proteinase on Duolite C-10 column. Column: 3 cm. in diameter and 80 cm. in height, 70-150 mesh of Duolite C-10 equilibrated with phosphate buffer of pH 6.5 containing 0.2 g. of Na^+ . Charge: 1.5 litre of dialyzed solution of crude commercial bacteria proteinase "Biopraxe" of about 2×10^5 $\mu\text{g.}$ of tyrosin unit per ml. Washing: Tap water, 20 per cent acetone, again tap water, finally 0.2 g. ion of Na^+ buffer of pH 7.0. Elution: 0.1 M NH_4OH , 0.1 M Na_2HPO_4 , 0.5 M NaCl mixture. Flow rate: 3-5 ml. per minute. Temperature: 8-10°.

centration of the salt. Thus, a displacement chromatography occurred and the first effluent of 3-5 volumes of the column capacity (hold-up volume) (Fraction I) was kept at pH 6-6.5 and contained no proteinase, no coloring matter, and a slight amount of impure proteins. When the eluting solution was still passed through and the upper dark-colored resin increased to some extent, concentrated proteinase suddenly appeared and thus about 70 per

cent of the adsorbed proteinase was eluted in a practically colorless solution (pH 6-6.5) of 2-4 hold-up volumes of the column (Fraction II). As the dark part of the column approached the lower end, the effluent became slightly colored, and the color increased gradually. On the contrary, the proteinase gradually decreased as the pH rose from 6.5 to 9.0 (about 1 hold-up volume, Fraction III). After all the resin became alkaline form, very dark-colored solution containing a slight amount of proteinase was obtained at pH 9 or higher (Fraction IV). A representative result obtained using *B. sub. N'* proteinase preparation as the source is shown in Fig. 3.

TABLE I
Summary of Purification and Crystallization of *B. subtilis N'* Proteinase

Fraction	Volume	Proteinase activity		Total activity		Purity		yield	
		PU ^{cas. 275, B} /ml. tyr.		PU ^{cas. 275, B} tyr.		PU/mg. PN		(%)	
		pH 7.5	pH 10	pH 7.5	pH 10	pH 7.5	pH 10	pH 7.5	pH 10
	ml.	x 10 ³	x 10 ³	x 10 ⁵	x 10 ⁵	x 10 ³	x 10 ³		
A. Extract of crude preparation	2420	7.93	7.06	192	171	5.10	4.65	100	100
B. Dialyzed solution before charge	2900	6.08	5.59	176	162	6.03	5.60	92	95
C. 1. Solution passed through	2900	0.450	0.567	13.0	16.4			6.8	9.6
2. 1st washings	950	1.20	1.43	11.4	13.6			5.9	8.0
3. 2nd washings (with acetone)	500	0.583	0.600	2.90	3.00			1.5	1.8
4. 3rd washings	625	0.013	0.016	0.080	0.090			0.04	0.05
5. Fraction I	312	0.052	0.061	0.160	0.190			0.08	0.11
6. Fraction II	464	16.5	17.5	76.5	81.3	9.47	10.4	40	48
7. Fraction III	130	5.54	6.25	7.20	8.10			3.8	4.8
8. Fraction IV	405	0.480	0.530	1.90	2.15			1.0	1.3
D. Solution of (NH ₄) ₂ SO ₄ precipitate from Fract. II	179	36.1	42.0	65.0	75.0	10.0	11.6	34	44
E. Solution of acetone precipitate	27.4	207	257	54.0	70.5	10.3	13.6	28	41
F. Solution of 1st crystals	21.2	251	309	53.0	65.5	11.3	13.7	28	38
G. Solution of 2nd crystals	16.2	286	346	46.2	56.0	11.3	13.7	24	33
H. Solution of 3rd crystals	17.2	255	308	43.6	52.8	11.4	13.8	23	31
I. Solution of 4th crystals	15.1	240	288	36.0	43.4	11.7	14.1	19	25

Essentially the same results were also obtained in the cases of other strains such as *B. sub. H, P, R*, and *N*.

In a similar experiment using the Scientific Reserch resin instead of Duolite C-10, the amount of proteinase adsorbed on the column was much less and the proteinase eluted in the fraction corresponding to the above Fraction II was lower in concentration on account of its poor adsorption power of proteinase and high capacity of taking the cations in the eluting solution. Separation of the coloring matter from the proteinase was worse than Duolite C-10 and the amount of colorless proteinase was much smaller.

Crystallization—Proteinase was conveniently concentrated from the above Fraction II by precipitation with ammonium sulfate and further by acetone precipitation. Since the enzyme was crystallized more easily from very concentrated solution than diluted one, the acetone precipitate was dissolved not by the addition of water but evaporation of the solvent. Usually small needle crystals appeared during or just after dissolving by evaporation and increased on standing, but it was better to crystallized by addition of a small amount of acetone after dissolving the enzyme completely. By the same way, almost pure and slightly colored crystals were also obtained from Fraction III in the eluates from the resin column. However, the color could not be completely removed from the crystals even by repeated recrystallizations. If it is necessary to increase the yield of crystals, it is better to repeat the resin treatment using smaller column or decoloring procedure using a special anion exchange resing such as Duolite A-2 or A-7 (20).

A representative results on the yield and purity of the proteinase in each step of purification and crystallization from preparation of *B. sub. N'* are shown in Table. I. The yield of the recrystallized preparation was about 3 g. from 100 g. of the commercial crude preparation and that of proteinase activity was 19 per cent by the assay at pH 7.5 and 25 per cent at pH 10.0.

This difference must be due to the presence of two kinds of proteinase, the enzyme having neutral optimum pH and alkaline pH, in the crude preparation as reported by Komaki (21). According to him, the neutral proteinase is very unstable and it was thought that this proteinase was completely inactivated in the process of resin treatment and only alkaline proteinase was obtained as crystals. Since the neutral proteinase is fairly active even at pH 10, the activity at pH 10.0 included this activity in addition to the activity of alkaline proteinase. Therefore, true yield of crystalline alkaline proteinase by the present procedure is thought to be larger than 25 per cent of the activity of the proteinase in the source material. In addition to the above fact, if the Fraction III were used for the crystallization, the yield will be somewhat larger.

The above method was also useful for the preparation of crystalline alkaline proteinase of *B. sub. P, H, R*, and *N*, and no marked difference in the results due to different source material was observed.

SUMMARY

A convenient method for the preparation of crystalline proteinase of *B. subtilis* using a cation exchange resin, Duolite C-10, was described. A solution of commercial enzyme preparation was passed through a column of the resin at neutral pH and, after washing the column, the adsorbed proteinase was eluted with alkaline solution containing a salt in high concentration in order to prevent elution of the coloring matter. From the colorless eluate, the enzyme was concentrated by salting out and acetone precipitation, and crystallized as needles or plates from acetone solution. The yield of the recrystallized enzyme was more than 25 per cent in activity.

The authors wish to express their many thanks to the Nagase & Co., Ltd., Osaka for the enzyme preparation and also to the Nippon Kiko Co., Ltd., Osaka, and The Scientific Reserch Institute Ltd. for the resins.

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TERMINAL OXIDATION SYSTEM IN BACTERIA

I. PURIFICATION OF CYTOCHROMES FROM *PSEUDOMONAS AERUGINOSA**

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Since Keilin (1) noted in 1925 that cytochromes are widely distributed in nature, reactions of the cytochrome system have been studied with baker's yeast (2, 3) and with a preparation of heart muscle extract (4, 5) devised by Keilin and Hartree (6). However, even with the use of animal tissues and baker's yeast, there has as yet been a great difficulty in reconstructing their cytochrome systems using all kinds of cytochromes purified and separated from each other, since the methods for purification have never been established except for a few cytochromes (7-10).

On the other hand, many workers (11-18) have attempted purification of bacterial cytochromes using several kinds of bacteria and reported that their cytochromes are not only essential for aerobic respiration, but also might have some roles in nitrate reduction (13-16) and sulfate reduction (17, 18).

This series of papers will deal with the cytochrome system of *Pseudomonas aeruginosa*, with a purpose of reconstructing the cytochrome system using all the cytochromes of this source separated from each other. In this paper will be described the purification of three kinds of water-soluble cytochromes and one kind of water-soluble, blue protein by a simple, continuous operation which consists of fractionation with ammonium sulfate and chromatography on an ion exchanger (Duolite CS-101) and activated aluminium oxide.

MATERIALS AND METHOD

Bacterial Strain and Culture—A strain of *Pseudomonas aeruginosa* was kindly supplied by Dr. Y. Takeda**. It was grown on a common bouillon-peptone agar (pH 7.0) at 37° for the purpose of its preservation. On use, it was cultured in bouillon-peptone medium at 37° for 24 hours with vigorous shaking, then 10 ml. of the cultured medium was poured into 2.5 liters of fresh bouillon-peptone medium and incubated at 37° for 24 hours with shaking. 10 liters of the incubated medium was transferred to a tank (50 cm. in diameter, 100 cm. in depth) which contained 100 liters of the following medium: Bouillon 1 kg.,

* This work was attempted and directed by K. Okunuki (same address to the author's).

** Research Institute for Microbial Diseases, University of Osaka.

peptone 1 kg., KNO_3 2 kg., KH_2PO_4 0.64 kg., and Na_2HPO_4 0.36 kg. (pH 6.5). This was incubated at 37° for 20 hours in static state. The pH was up to about 8.2 at the end of the incubation. The grown cells were collected by a Sharpless centrifuge or by filtration with the aid of Hyflo Super Cel. The higher pH at the end caused the grown cells to be autolyzed and some cytochromes to be lost in the filtrate.

Preparation of Acetone Powder of the Cells—The cells collected by centrifugation or together with Hyflo Super Cel by filtration were gradually thrown into 5 liters of acetone previously chilled to -5° and were filtered after 10 minutes' stirring. The debris was again washed with 2.5 liters of the chilled acetone and dried over CaCl_2 in a vacuum desiccator.

Adjustment of Ion Exchanger—Cation exchanger (Duolite CS-101), 100–150 mesh in Na-form, was activated by N NaOH and then by N HCl as for the purification of cytochrome *c* from baker's yeast (19). On use, the activated resin was buffered with 0.1 M ammonium phosphate buffer solution (pH 5.7 and pH 6.0). Concentration of this buffer solution will be expressed below by that of ammonium ion.

Adjustment of Activated Aluminium Oxide—Commercial activated aluminium oxide for chromatography* (200 mesh) was boiled for 15 minutes in N NaOH. After being washed several times with water, the aluminium oxide was mixed with an equal volume of sodium hypochlorite solution (3.5 per cent) and allowed to stand overnight. After being washed thoroughly with water, it was immersed in N HCl and stirred often. On use, after thorough washing with water, the reactivated aluminium oxide was buffered with 0.1 M ammonium phosphate buffer solution (pH 5.9–6.0). The exhausted aluminium oxide was regenerated by the same treatment as described above.

Measurement of Absorption Spectrum—In purification steps, the cytochromes were confirmed using a hand-spectroscope. Quantitative determination was performed by Shimadzu photoelectric spectrophotometer, type QB-50, and Beckman ratio recording spectrophotometer.

Fractionation with Ammonium Sulfate—Solid ammonium sulfate was used and all of fractionation was performed in pH-range between 6.0 and 7.0.

Dialysis—This was carried out with a cellophane pouch (about 50 ml. in capacity) against water below 5° .

RESULTS AND DISCUSSION

Extraction of Cytochromes from Acetone Powder—The acetone powder of *Pseudomonas aeruginosa* was mixed little by little with ten volumes of 0.1 M sodium citrate buffer solution (pH 6.4) or 0.1 M sodium phosphate buffer solution (pH 6.5), blended sufficiently in a mortar, and incubated at 40° for 15 minutes. After standing overnight in a refrigerator, the extract was separated from the debris by filtration on Büchner funnel. The same extraction was repeated twice more. The extract was a little turbid, fairly viscous, and brown coloured. These extracting conditions were selected after comparison with various treatments as shown in Table I.

Removal of Viscous Substances from Extract—To the extract 2 per cent acrinol solution was added until fibrous matter was no longer formed. Most of the matter was taken out using a net covered with gauze and the remainder was separated from the solution by filtration.

* E. Merck Chemical Works, and Wako Pure Chemical Industries, Ltd.

TABLE I

Extracting Conditions for Pseudomonas Cytochromes

1) Effect of the kind of salt solution*.

Conditions				Intensity of absorption	
Salt	Concentration	pH	Temp. (°C)*	Na-dithionite	
				Non	Added
H ₂ O	—	—	40	+	+++
(NH ₄) ₂ SO ₄	10% in satn.	7.0	"	±	++
"	20% in satn.	"	"	±	++
Na-phosphate	0.1 M	"	"	+	+++
"	0.33 M	"	"	±	++
Na-citrate	0.1 M	"	"	+++	+++
"	0.3 M	"	"	++	++

2) Effect of pH*.

Na-phosphate	0.1 M	5.0	40	+	+++
		6.2	"	++	+++
		7.3	"	++	+++
		7.9	"	++	+++
		8.4	"	+	++
Na-citrate	0.1 M	5.0	"	++	++
		6.4	"	+++	+++
		7.1	"	+++	+++
		7.9	"	++	+++
		8.5	"	++	+++

3) Effect of temperature*.

Na-citrate	0.1 M	7.0	10		++
			20		++
			30		+++
			40		+++
			50		+
			60		±

* Ten ml. of each solution was added to 500 mg. of acetone powder of bacterial cells and homogenized well in a mortar. After the treatment shown in the Table, the suspension was centrifuged and the intensity of absorption band at about 545–555 mμ of the supernatant solution was compared to each other by a hand-spectroscope.

** After heating at each temperature for 15 minutes, allowed to stand overnight in a refrigerator.

Precipitation of Cytochromes with Ammonium Sulfate—Solid ammonium sulfate was added to the solution to 35 per cent in saturation. The precipi-

tate was filtered off. The filtrate was supplemented with ammonium sulfate to 90–95 per cent in saturation to form a viscous precipitate. The precipitated cytochrome was collected by filtration and transferred into cellophane pouches using a small amount of water and the pouches were dialyzed against water for one day. The inner solution was clarified by centrifugation.

Purification of Cytochromes by Cation Exchanger—The clear solution was passed through a column (1.5×35 cm.) packed with Duolite CS-101 (pH 5.7). Acrinol, which had not been removed by the dialysis, was adsorbed on the top of the column and just below, greenish brown substance was adsorbed, while most of the cytochrome failed to be adsorbed. The adsorbed substance was eluted with 2 *M* ammonium phosphate buffer solution (pH 7.0) and separated from the acrinol which was not eluted. The greenish brown eluate was again precipitated with ammonium sulfate and dialyzed against water for 24 hours. After centrifugation, the dialyzed solution was again passed through a column packed with Duolite CS-101 (pH 6.0) to the same extent as for the first column. The effluent contained a cytochrome which exhibited an absorption band at 554 m μ as its α -band, if reduced by dithionite. However, a small part of this cytochrome was adsorbed together with the greenish brown substance on the cation exchanger even under this condition; the cytochrome was more weakly adsorbed on the resin at pH 6.0 than at 5.7. The cytochrome of α -band at 554 m μ will be denoted as cytochrome₅₅₄ in the following sentences. When the charged column was washed with water and eluted with 2 *M* ammonium phosphate buffer solution (pH 7.0), the greenish brown substance was completely eluted together with a small amount of cytochrome₅₅₄, while many brown impurities remained in the column. All of the greenish brown substances was precipitated by 40 to 60 per cent ammonium sulfate in saturation and could be completely separated from the cytochrome₅₅₄, since the latter was precipitated at 80–95 per cent in saturation. Further, this substance could be purified by chromatography on the CS-101 column buffered with 0.1 *M* ammonium phosphate buffer solution (pH 6.0), on which the substance previously dialyzed overnight against the same buffer solution was charged and developed by the same buffer solution. The greenish brown substance had in its reduced state a broad absorption band at 620–630 m μ and three narrow bands at 551, 548, 521–523 and 418 m μ , as shown in Fig. 1-a and -b. In its oxidized state, the broad band at 620–630 m μ scarcely changed but the band at 418 m μ shifted to 409 m μ , and the other three bands disappeared, while two broad but weak bands appeared at 560–570 m μ and 520–540 m μ . This greenish brown substance (cytochrome_{GB}) had a strong autooxidizability.

Purification of Cytochromes by Aluminium Oxide—The effluent from the first cation exchanger was precipitated by 95 per cent ammonium sulfate in saturation and the precipitate was dialyzed against water for 24 hours. After centrifugation, the inner solution was passed through a column packed with aluminium oxide in the same scale as described above. The coloured

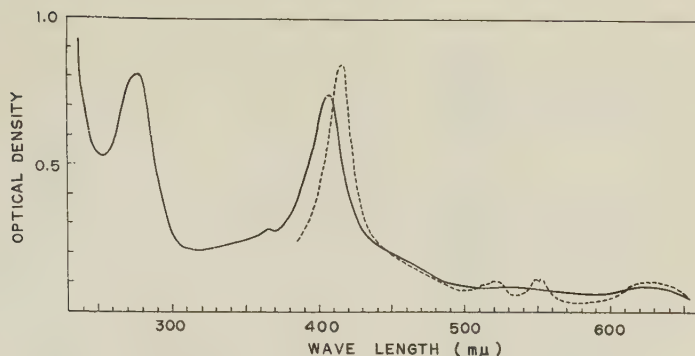


FIG. 1-a. Absorption spectra of *Pseudomonas* cytochrome_{GB}.
Solid line, in oxidized form; broken line, in reduced form.

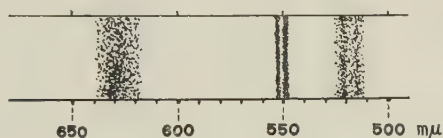


FIG. 1-b. Diagrammatic representation of visible absorption
bands of *Pseudomonas* cytochrome_{GB}.

Observed in its reduced form with use of a hand-spectroscope.

substances present in the inner solution were adsorbed on the top of the column. However, when the charged column was washed with water, the cytochrome gradually moved down, brown impurities remaining on the top. When the fraction of cytochromes was completely separated from the impurities, the two were separated by scraping away the top part of the column with the impurities. This chromatography was remarkably advantageous in purifying cytochromes, but it took a long time for elution. In practice, the scraped column was once eluted with 2 *M* ammonium phosphate buffer solution (pH 7.0) and the eluate was precipitated by 95 per cent ammonium sulfate in saturation. The precipitate was dialyzed together with the cytochrome₅₅₄ fraction in the above section against water for 24 hours and then against 0.1 *M* ammonium phosphate buffer solution (pH 5.9–6.0) for 15 hours. The inner solution, after centrifugation, was passed through the column packed to the same extent as described above with aluminium oxide buffered with the buffer solution and elution of the charged substances was performed with the same buffer solution. The manner of the elution was shown in Figs. 2 and 3. Yellowish brown matter was first eluted out and removed, and after some intervals, red fraction and blue fraction were eluted and fractionated. As shown in Fig. 3, these two kinds of coloured substances were eluted one after the other in most of the fractions. The two could be easily and completely separated, since the red substance was precipitated by 60–75 per cent saturation of ammonium sulfate, while the blue substance by 85–95 per cent. The former had α -band at 551 *mμ* in its reduced state

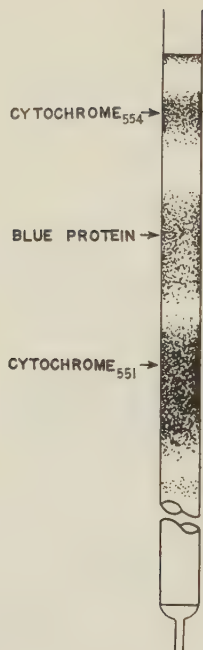


FIG. 2. Chromatogram of the *Pseudomonas* cytochromes on aluminium oxide.

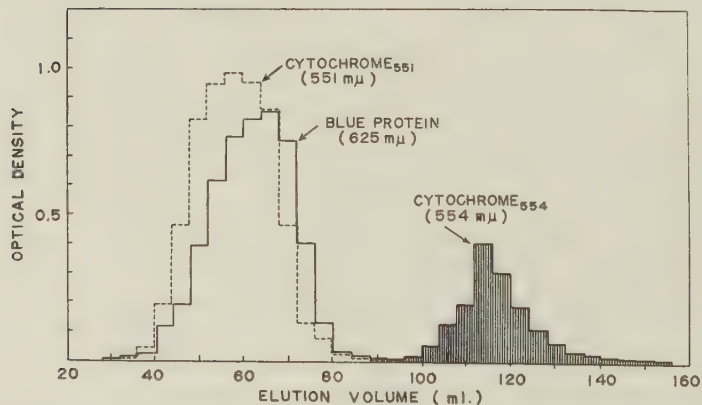


FIG. 3. Chromatographic fractionation of the *Pseudomonas* cytochromes on aluminium oxide.

(cytochrome₅₅₁) and the latter had a broad band in the neighbourhood of 630 mμ in its oxidized state (blue protein). After them, another red substance was slowly eluted and was completely separated from both of the foregoing proteins. This was cytochrome₅₅₁. Absorption spectra of cytochrome₅₅₁, blue protein, and cytochrome₅₅₄ were measured at this stage by Mr. Fujino* using the Backman ratio recording spectrophotometer as

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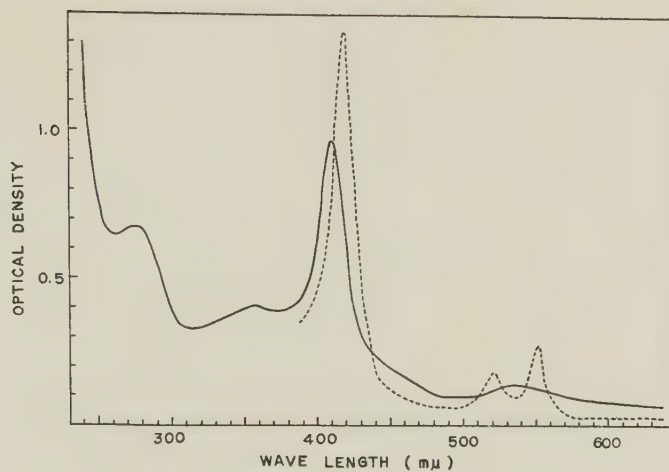


FIG. 4. Absorption spectra of *Pseudomonas* cytochrome c_{551} . Solid line, in oxidized form; broken line, in reduced form.

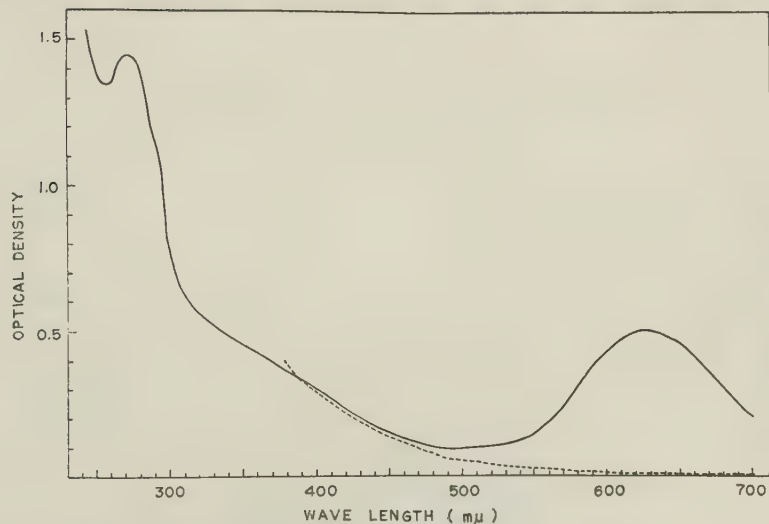


FIG. 5. Absorption spectra of *Pseudomonas* blue protein. Solid line, in oxidized form; broken line, reduced form.

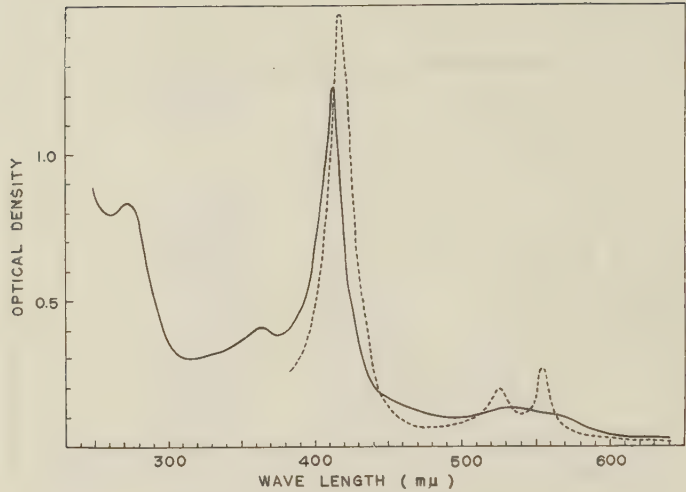


FIG. 6. Absorption spectra of *Pseudomonas* cytochrome₅₅₄. Solid line, in oxidized form; broken line, in reduced form.

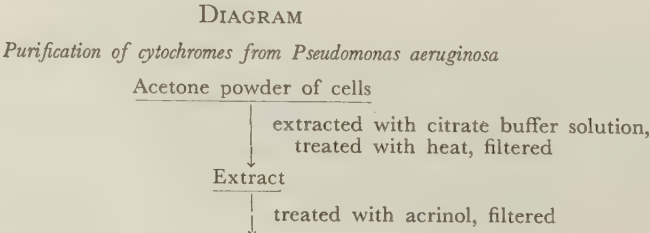
shown in Figs. 4, 5, and 6.

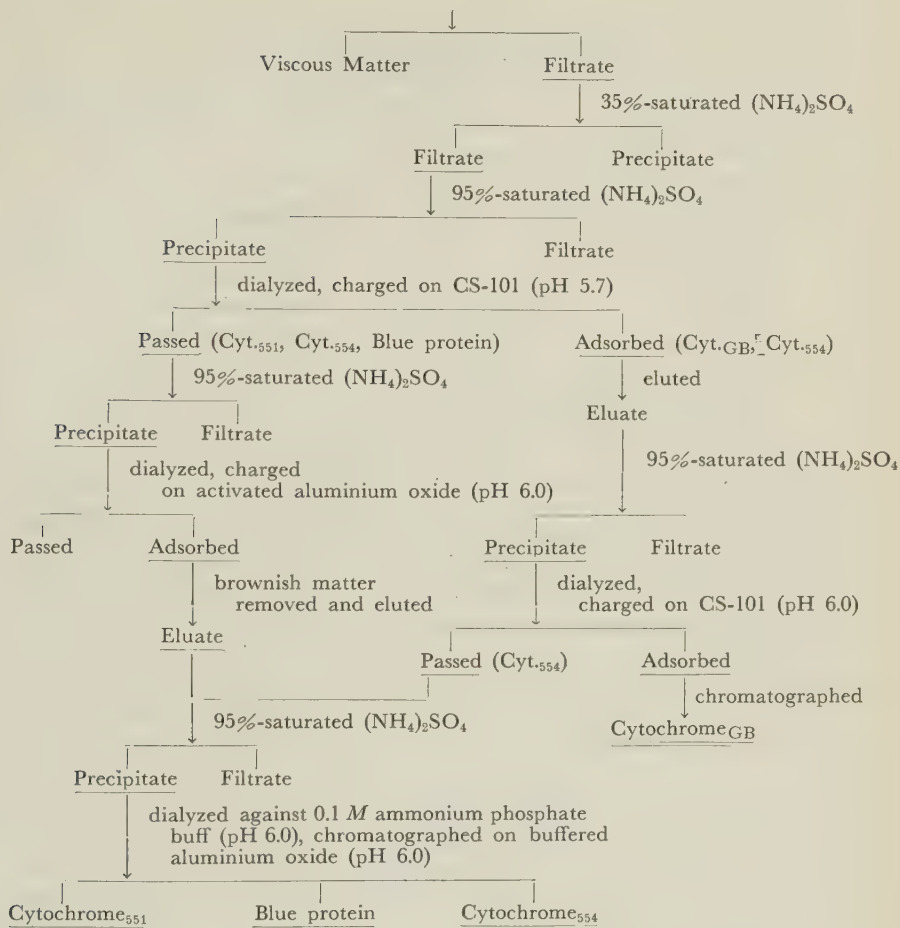
These bacterial proteins were further purified by repeating aluminium oxide chromatography in their oxidized and reduced state, under the conditions described above. The behaviour of these proteins to salting-out by ammonium sulfate and to other treatments are summarized in Table II,

TABLE II
Comparison of Purified Cytochrome for Various Treatments

Pigments	Salting out by (NH ₄) ₂ SO ₄ (% in saturation)	Adsorption on Duolite CS-101	Adsorption on aluminium oxide
Cytochrome ₅₅₄	80-95	+	++
Cytochrome ₅₅₁	60-75	-	++
Cytochrome _{GB}	40-60	++	+++
Blue protein	85-95	±	++

and the procedure for purification is summarized in the Diagram.





Other Cytochromes in the Bacteria—The paste of the living cells of this bacteria was pink-coloured. According to observations by a hand-spectroscope, it seemed likely that there were two or three other cytochromes, not purified by the continuous operation as described above, which might be of the so-called a- and b- groups, as shown in Fig. 7. and Table III.

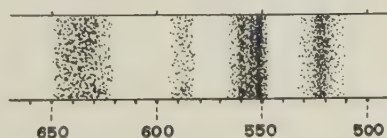


FIG. 7. Diagrammatic representation of visible absorption bands of the living cells of *Pseudomonas aeruginosa*.

Observed by a hand-spectroscope.

TABLE III
Cytochromes of Pseudomonas aeruginosa

Cytochromes	α -Absorption band in reduced state (m μ)*
Cytochrome a ₂ -like	630**
Cytochrome a ₁ -like	590**
Cytochrome b ₁ -like	560**
Cytochrome ₅₅₄	554
Cytochrome ₅₅₁	551
Cytochrome _{GB}	620-630, 551, 548
Blue protein	(625) in oxidized state

* Observed with use of a hand-spectroscope.

** Observed in vivo.

Extraction of Cytochromes by the Use of Deoxycholate—The paste of the living cells was ground with two-fold weight of quartz sand for one hour in a mortar cooled by salt-ice bath. Then the ground cells were extracted overnight in a refrigerator with ten volumes of 10 per cent saturation of ammonium sulfate solution containing 1 per cent of deoxycholate (pH 7.0) and centrifuged at 13,000 r.p.m. for 20 minutes using a Servall centrifuge. The supernatant appeared to contain most of the cytochromes which had been observed in the bacterial paste by a hand-spectroscope. Namely, there might be two or three cytochromes which belong to a- and b- groups other than the cytochromes purified as described above. When the ground cells were extracted with 0.1 M sodium phosphate buffer solution (pH 7.0), a fairly large amount of cytochrome₅₅₁ and a small amount of cytochrome₅₅₄ were extracted, but the other cytochromes were hardly extracted. Compared with extraction from the acetone powder, this extraction from the ground cells appeared not to be advantageous for the preparation, since the extract from the ground cells contained much more impurities than that from the acetone powder. Purification of the cytochromes extracted only by the use of deoxycholate is being studied.

SUMMARY

Water-soluble cytochromes from *Pseudomonas aeruginosa* have been purified by a simple, continuous procedure which consists of chromatographies on an ion exchanger, Duolite CS-101, and aluminium oxide, and ammonium sulfate fractionation. A cytochrome adsorbed on CS-101 has the absorption maxima at 620-630, 551, 548, 521-523, and 418 m μ in the reduced state (*Pseudomonas* cytochrome_{GB}), and two cytochromes purified by chromatography on aluminium oxide have the absorption maxima at 551, 521, and 416 m μ (*Pseudomonas* cytochrome₅₅₁), and at 554, 525, and 416 m μ (*Pseudomonas* cytochrome₅₅₄), in the reduced state, respectively. Further, a blue-coloured protein having an absorption maximum at 625 m μ in the oxidized

state was purified (*Pseudomonas* blue protein) and when this protein was reduced, the absorption maximum disappeared. Attempts were made to extract other cytochromes present in the bacteria by other procedures.

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LETTERS TO THE EDITORS

The Journal of Biochemistry, Vol. 45, No. 3, 1958

FURANOSE STRUCTURE OF PARA-AMINO-BENZOYLGLUCURONIDE ISOLATED FROM DOG URINE

Sirs:

All of the glucuronides found in nature were believed to be a pyranose type, but *p*-aminobenzoylglucuronide isolated from dog urine after administration of *p*-aminobenzoic acid (PABA) is most likely to have a furanose configuration. The procedure employed for the isolation of this glucuronide was similar to that of Bray *et al.* and the physical constants are as follows. White needles, m.p. 187° (decomp.) (ammonium salt).

Analysis. Calcd. for $C_{13}H_{18}O_8N_2 \cdot H_2O \frac{1}{2} CH_3COCH_3$ (ammonium salt);

C, 46.2; H, 6.10; N, 7.43 per cent

Found; C, 46.34; H, 5.98; N, 7.58 per cent

Glucuronic acid (by Jarrige's method) found/calcd. 96 per cent. Reducing power (by Hagedorn-Jensen's method) found/calcd. 97 per cent. PABA (Tsuda's diazo coupling method) found/calcd. 98.5 per cent. PABA (ultraviolet absorption after hydrolysis with Na_2CO_3 solution) found/calcd. 100.5 per cent. Infrared spectrum showed a strong absorption

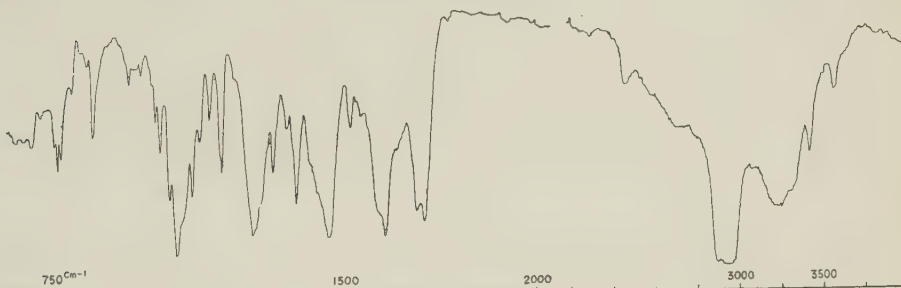


FIG. 1-a. Infrared spectrum of *p*-aminobenzoyl-glucuronide (free form).

due to ester carbonyl in the region of 1680 cm^{-1} . Moreover, when PABA-glucuronide was diazotized and coupled with Tsuda's reagent, *N*-(diethylaminoethyl)-naphthylamine, the resulting product was not transferred to ether, in contrast to uncoupled PABA. From these facts, it was concluded that this glucuronide was O-glucuronide. The infrared absorption spectrum of PABA-glucuronide as well as of its ammonium salt (Fig. 1 a and b) showed neither of type 2a nor 2b, indicative of pyranose configuration but typical bands of furanose in the regions of 940 cm^{-1} , 830 cm^{-1} and 790 cm^{-1} (and 880 cm^{-1} as a shoulder).

In order to verify the furanose configuration, following experiment was carried out. Ten milliliters of 0.001 *N* solution of the glucuronide was oxidized with 0.01 *N* periodic acid for 24 to 48 hours, then the mixture



FIG. 1-b. Infrared spectrum of *p*-aminobenzoyl-glucuronide (ammonium salt).

was neutralized with barium hydroxide in order to remove excess periodic and iodic acid. To the supernatant was added an additional barium hydroxide, the solution was hydrolysed at 100° for several minutes and then passed through a column of Amberlite IR-120 (H⁺) to remove the aglycone and barium ion. The effluent was then treated in the following two different ways. (A) The effluent was refluxed with about 30 mg. of yellow mercury oxide for 30 minutes and filtered while still hot. Mercury in the filtrate was removed by precipitation with hydrogen sulphide and centrifugation. The supernatant was concentrated under a reduced pressure (Sample a). (B) Thirty mg. of calcium carbonate and a drop of bromine were added to the above effluent. The mixture was left at room temperature for 30 minutes with occasional shaking. Then nitrogen gas was passed through the solution to drive off the excess bromine and the calcium ion was removed by Amberlite IR-120 (H⁺). The supernatant liquid was concentrated under a reduced pressure (Sample b). Both samples (a) and (b) were submitted to paper chromatography and sprayed with methylred-alcohol solution to detect the spots of the products. The results are demonstrated in Table I.

TABLE I

Paper Chromatography of the Oxidation Products of PABA-Glucuronide

Solvent	Oxalic acid	Saccharolactone	Glucuronic acid	Tartroic acid	D-Tartaric acid	Mesotartaric acid	Sample (a)	Sample (b)
Butanol, acetic acid, water; 4:1:5	0.40 tail	0.41	0.15	0.35	0.38	0.30	0.30	0.27
Butanol, acetic acid, water; 2:2:1	0.60	0.44	0.41	0.58	0.52	0.50	0.50	0.50
Ethyl acetate, acetic acid water; 3:1:1	0.45 tail	0.50	0.15	0.38	0.40	0.26	0.22	0.23
Ether, acetic acid, water; 13:3:1	0.30 tail	0.15	0.00	0.27	0.15	0.03	0.02	0.03
Phenol, formic acid, water; 75:1:25	0.36	0.70	0.24	0.31	0.26	0.25	0.25	0.25
Ethanol, conc. ammonia, water; 8:1:1	0.02	0.35	0.35	0.06	0.20	0.60	0.58	0.58

R_f value of the (samples (a) and (b) agreed very well with those of a known sample of mesotartaric acid. The results lead us to the conclusion that PABA-glucuronide might have a furanose configuration and the aglycone might be conjugated to C-1 of glucuronic acid, as recognized before (4-6).

The authors are indebted to Dr. G. Chihara for the infrared absorption spectroscopy.

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